



THE JOURNAL OF  
GENERAL PHYSIOLOGY





# THE JOURNAL OF GENERAL PHYSIOLOGY

EDITORS

JACQUES LOEB

W. J. V. OSTERHOUT

VOLUME FIFTH  
WITH 1 PLATE AND 244 FIGURES  
IN THE TEXT



NEW YORK  
THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH  
1923

COPYRIGHT, 1923, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

WAVERLY PRESS  
THE WILLIAMS & WILKINS COMPANY  
BALTIMORE, U. S. A.

# CONTENTS.

No. 1, SEPTEMBER 20, 1922.

HECHT, SELIG, and WILLIAMS, ROBERT E. The visibility of monochromatic radiation and the absorption spectrum of visual purple.....	1
HITCHCOCK, DAVID I. The colloidal behavior of serum globulin.....	35
PARKER, G. H. The excretion of carbon dioxide by relaxed and contracted sea anemones.....	45
CROZIER, W. J. Cell penetration by acids. VI. The chloroacetic acids.....	65
ARRHENIUS, OLOF. Absorption of nutrients and plant growth in relation to hydrogen ion concentration.....	81
LOEB, JACQUES. Cataphoretic charges of collodion particles and anomalous osmosis through collodion membranes free from protein.....	89
LOEB, JACQUES. The influence of electrolytes on the cataphoretic charge of colloidal particles and the stability of their suspensions. I. Experiments with collodion particles.....	109

No. 2, NOVEMBER 20, 1922.

DE KRUIF, PAUL H., and NORTHROP, JOHN H. The stability of bacterial suspensions. IV. The combination of antigen and antibody at different hydrogen ion concentrations.....	127
DE KRUIF, PAUL H., and NORTHROP, JOHN H. The stability of bacterial suspensions. V. The removal of antibody from sensitized organisms.....	139
FENN, WALLACE O. The adhesiveness of leucocytes to solid surfaces.....	143
FENN, WALLACE O. Effect of the hydrogen ion concentration on the phagocytosis and adhesiveness of leucocytes.....	169
JACOBS, M. H. The influence of ammonium salts on cell reaction.....	181
CHAMBERS, ROBERT. A micro injection study on the permeability of the starfish egg.....	189

MEEK, CAROLYN S., and LIPMAN, CHARLES B. The relation of the reaction and of salt content of the medium on nitrifying bacteria. ....	195
BRODY, SAMUEL, and RAGSDALE, ARTHUR C. The equivalence of age in animals. ....	205
HARVEY, E. NEWTON. The permeability of cells for oxygen and its significance for the theory of stimulation. ....	215
IRWIN, MARIAN. The permeability of living cells to dyes as affected by hydrogen ion concentration. ....	223
OSTERHOUT, W. J. V. Some aspects of selective absorption. ....	225
LOEB, JACQUES. Sodium chloride and selective diffusion in living organisms. ....	231
LOEB, JACQUES. The influence of salts on the rate of diffusion of acid through collodion membranes. ....	255
NORTHROP, JOHN H. The mechanism of the influence of acids and alkalies on the digestion of proteins by pepsin or trypsin. ....	263

### NO. 3, JANUARY 20, 1923.

HARVEY, E. NEWTON. Studies on bioluminescence. XV. Electroreduction of oxyluciferin. ....	275
WAKSMAN, SELMAN A., and STARKEY, ROBERT L. On the growth and respiration of sulfur-oxidizing bacteria. ....	285
FENN, WALLACE O. The phagocytosis of solid particles. IV. Carbon and quartz in solutions of varying acidity. ....	311
MOORE, A. R. Muscle tension and reflexes in the earthworm. ....	327
HUSSEY, RAYMOND G., and NORTHROP, JOHN H. A study of the equilibrium between the so called "antitrypsin" of the blood and trypsin. ....	335
NORTHROP, JOHN H., and HUSSEY, RAYMOND G. A method for the quantitative determination of trypsin and pepsin. ....	353
HUSSEY, RAYMOND G. Further observations on the influence of salts when injected into the animal body. ....	359
BROOKS, S. C. Conductivity as a measure of vitality and death. ....	365
HITCHCOCK, DAVID I. The ionization of protein chlorides. ....	383
LOEB, JACQUES. The influence of electrolytes on the cataphoretic charge of colloidal particles and the stability of their suspensions. II. Experiments with particles of gelatin, casein, and denatured egg albumin. ....	395

NORTHROP, JOHN H. The mechanism of the effect of acids and alkalis on the digestion of proteins by pepsin or trypsin. A correction.....	415
---	-----

#### No. 4, MARCH 20, 1923.

COLE, WILLIAM H. Circus movements of <i>Limulus</i> and the tropism theory.....	417
IRWIN, MARIAN. The behavior of chlorides in the cell sap of <i>Nilella</i> .....	427
DU NOÛY, P. LECOMTE. A new viscometer.....	429
BRODY, SAMUEL, RAGSDALE, ARTHUR C., and TURNER, CHARLES W. The rate of decline of milk secretion with the advance of the period of lactation.....	441
BRODY, SAMUEL, RAGSDALE, ARTHUR C., and TURNER, CHARLES W. The rate of growth of the dairy cow. II. Growth in weight after the age of two years .....	445
MOORE, A. R. The reaction of <i>Nereis virens</i> to unilateral tension of its musculature.....	451
MOORE, A. R. Galvanotropism in the earthworm.....	453
STANTON, RALPH E. The selective absorption of potassium by animal cells. III. The effect of hydrogen ion concentration upon the retention of potassium.....	461
RAY, GEORGE B. Comparative studies on respiration. XXIV. The effects of chloroform on the respiration of dead and of living tissue.....	469
LOEB, JACQUES. Stability of suspensions of solid particles of proteins and protective action of colloids.....	479
LOEB, JACQUES. Membrane potentials and cataphoretic potentials of proteins.....	505

#### No. 5, MAY 20, 1923.

COHN, EDWIN J., and HENDRY, JESSIE L. Studies in the physical chemistry of the proteins. II. The relation between the solubility of casein and its capacity to combine with base. The solubility of casein in systems containing the protein and sodium hydroxide.....	521
--	-----

HECHT, SELIG. Sensory adaptation and the stationary state....	555
NORTHROP, JOHN H., and LOEB, JACQUES. The photochemical basis of animal heliotropism.....	581
CROZIER, W. J., and MOORE, A. R. Homostrophic reflex and stereotropism in diplopods.....	597
NORTHROP, JOHN H. The stability of bacterial suspensions. VI. The influence of the concentration of the suspension on the concentration of salt required to cause complete agglutination.	605
RAY, GEORGE B. Comparative studies on respiration. XXV. The action of chloroform on the oxidation of some organic acids.....	611
RAY, GEORGE B. Comparative studies on respiration. XXVI. The production of CO <sub>2</sub> from organic acids in relation to their iodine absorption.....	623
HOAGLAND, D. R., and DAVIS, A. R. The composition of the cell sap of the plant in relation to the absorption of ions.....	629
HUSSEY, RAYMOND G., and THOMPSON, WILLIAM R. The effect of radio-active radiations and x-rays on enzymes. I. The effect of radiations from radium emanation on solutions of trypsin.	647
HITCHCOCK, DAVID I. Membrane potentials in the Donnan equilibrium.....	661
LOEB, JACQUES, and KUNITZ, M. Valency rule and alleged Hofmeister series in the colloidal behavior of proteins. I. The action of acids.....	665
LOEB, JACQUES, and KUNITZ, M. Valency rule and alleged Hofmeister series in the colloidal behavior of proteins. II. The influence of salts.....	693

### No. 6, JULY 20, 1923.

OSTERHOUT, W. J. V. Exosmosis in relation to injury and permeability.....	709
IRWIN, MARIAN. The penetration of dyes as influenced by hydrogen ion concentration.....	727
RAY, GEORGE B. Comparative studies on respiration. XXVII. The mechanism of oxidation in relation to chloroform anesthesia.....	741

NORTHROP, JOHN H. Note on the purification and precipitation of casein.....	749
NORTHROP, JOHN H. The inactivation of trypsin. IV. The adsorption of trypsin by charcoal.....	751
SPONSLER, O. L. Structural units of starch determined by x-ray crystal structure method.....	757
BRODY, SAMUEL, RAGSDALE, ARTHUR C., and TURNER, CHARLES W. The effect of gestation on the rate of decline of milk secretion with the advance of the period of lactation.....	777
SHACKELL, L. F. Studies in protoplasm poisoning. I. Phenols.	783
LILLIE, R. S., and CATTELL, WARE. The relation between the electrical conductivity of the external medium and the rate of cell division in sea urchin eggs.....	807
CHAMBERS, ROBERT, and SANDS, HAROLD C. A dissection of the chromosomes in the pollen mother cells of <i>Tradescantia virginica</i> L. Plate 1.....	815
CHAMBERS, ROBERT. The mechanism of the entrance of sperm into the starfish egg.....	821
LOEB, JACQUES. Theory of regeneration based on mass action..	831
LOEB, JACQUES. Theory of geotropism based on mass action...	853
INDEX TO VOLUME V.....	865





# THE VISIBILITY OF MONOCHROMATIC RADIATION AND THE ABSORPTION SPECTRUM OF VISUAL PURPLE.

By SELIG HECHT\* AND ROBERT E. WILLIAMS.

(From the Laboratory of Physical Chemistry, Harvard Medical School, Boston, and  
the George Holt Physics Laboratory, University of Liverpool, Liverpool.)

(Received for publication, July 1, 1922.)

## I.

### *Reasons for Experiments.*

When the visible spectrum is reduced to a very low intensity, and is viewed by a dark adapted eye, it appears colorless. The different portions of the spectrum, however, possess different degrees of brightness, the center being much brighter than the ends. Apparently, a given quantity of light energy will produce a quantitatively different effect depending on its frequency. It seemed to us that an exact knowledge of the relation between frequency and brightness at low intensities of illumination should furnish data bearing on the mechanism of dim vision, a hint of which had already been received in a study of dark adaptation (Hecht, 1919-20). We therefore set ourselves the task of determining accurately this relation between the frequency of light and its ability to produce a colorless sensation in the eye.

Attempts to do precisely this have already been made by several investigators. Perhaps the earliest were Ebert (1888), Langley (1888) and Hillebrand (1890). They were followed soon afterward by Abney and Festing (1893) and von Kries and Nagel (1896), and later by Schatarnikoff (1902), Pflüger (1902) and Trendelenburg (1904). It might therefore seem superfluous for us to have undertaken this work, and to add another to the already overwhelming number of papers on vision. Unfortunately, however, the work of the above mentioned investigators is far from adequate, first because of the failure to recog-

\* National Research Fellow.

nize certain factors entering into the problem, and second because of the sparse and irregular nature of the data.

It seems quite obvious that in order to make any correct comparisons of the relative effects and stimulating power of the different portions of the spectrum, it is necessary to know their energy content. Yet Langley and Pflüger were the only investigators who took this into consideration, and actually measured the energy distribution of the light which they used. The remaining authors either seemed unaware of this point (Ebert, Hillebrand, Abney and Festing, von Kries and Nagel, and Schatarnikoff), or being aware, made no measurements with which to correct their data (Trendelenburg). Schatarnikoff, for example, determined the relation between wave-length and brightness for the spectrum of gas light, of sunlight, and of diffuse daylight, and notes without further comment that the maxima are in different positions. Pflüger's results, though corrected for energy distribution, are so irregular that they contribute very little to our knowledge except that light between 495 and 525 $\mu$  possesses the maximum capacity for stimulating the dark adapted eye. Langley's results, though much better, are too few to serve our purpose, his measurements having been made with only three or four individuals.

A second factor that enters into the measurements is again ignored by all the investigators except Langley and Pflüger. In making determinations of the relative stimulating power of different lights it is necessary to make comparisons of the intensities which will produce the *same* effect in the eye. In other words, one must find the amount of light at different frequencies which will produce the same brightness in the eye. Both Langley and Pflüger determined the energy at the threshold of visibility. The other investigators kept neither the brightness nor the energy constant, but varied both during the experiments.

In short, it seemed high time to secure results by a method which should not only be free from the errors previously committed, but which should be applicable for use with a large number of individuals so as to yield data sufficient to establish the relationship for the eye in general.

Such data are urgently necessary at this time. Ever since Koenig's (1894) first determination of the absorption spectrum of visual purple, there has been a growing conviction, stimulated by the work of Trendelenburg (1904) and the publicity of Henri and Languier des Bancel's (1911), that visual purple is the receptor substance for dim or twilight vision. The main point in the evidence has been that the absorption spectrum and the velocity of bleaching of visual purple coincide with the stimulating power of spectral radiation at low intensities. The degree of coincidence of these three phenomena is, however, far from convincing. Due to the inadequacy of the data, their rather large deviations may be ascribed to individual variation, and to the necessity for making energy distribution corrections in the available data. These matters, however, lie at the very root of the photochemistry of photoreception, and cannot be dismissed in such an off hand manner. They demand an accurate, quantitative investigation.

This we have endeavored to do. As will be apparent, the effort has been amply repaid by the demonstration of new regularities and suggestive correlations, quite different from what would be expected in terms of the inadequate data heretofore available.

## II.

### *Apparatus, Methods, and Calibrations.*

1. The method which we first used consisted in determining the minimum intensity of a series of monochromatic illuminations which could just be perceived by the periphery of the eye. The apparatus was essentially a spectrometer, the light from which, after passing through a pair of Nicol prisms, was spread out on a plate of ground glass. Although we used highly intelligent subjects, our results were irregular. They resembled the data published by Pflüger, and for our purposes were flat failures.

The causes of the failure of such a method are instructive. In the first place, the experiments take a certain length of time, and during that time the eye of the subject varies. We do not speak of dark adaptation—a source of error which we recognized and carefully controlled. We refer more to the normal variations in the visual

mechanisms from minute to minute, and to the effects of the strain involved in looking for these extremely dim lights. These variations involve changes of 100 per cent or more, and with this method there is no way of controlling them or of compensating for them. In the second place, it is extremely difficult to tell when one sees a very dim light and when one does not; and to be able to indicate the exact stage of the intensity when a light first appears or ceases to exist is a taxing task, especially if it has to be done a dozen times with different portions of the spectrum. After our experience, Pfüger's irregular results are not surprising. Our results with this method are easily as erratic as those of Pfüger.

The method that we eventually adopted eliminated both these sources of variation. Instead of working with illuminations which are only just perceptible, we used an illumination which, though well below the color threshold, is still bright enough to be seen easily by the dark adapted eye. We then measured the relative energy at the various frequencies which is necessary to produce this degree of brightness. Our method is therefore a photometric one, in which one of the lights is kept at a constant brightness. In this way there is at once removed the uncertainty and strain of looking for lights that are not there. Moreover, there are eliminated the effects of variation in sensitivity of the visual system during the course of the experiments. The subject is required to match photometrically the constant light which serves as a standard. If the eye varies, it does so both for the standard and for the experimental light at the same time. With this method, the results took on at once a gratifying smoothness and regularity which will be apparent in the data to be presented.

2. The arrangement of the apparatus will be made clear with the help of Fig. 1. A 500 candle power Pointolite lamp in a light-tight box has its incandescent target focussed on the slit of a Hilger monochrometer. This is a spectrometer of the constant deviation type with a slit and thermopile groove in place of the eyepiece. In these experiments both slits of the spectrometer were kept at a width of 0.1 mm. The monochromatic light coming from the spectrometer spreads out gradually into a broad beam. Before it has sensibly diverged, however, it is passed through a pair of nicols, taken with

their mountings from a Hilger spectrophotometer. The beam then spreads out, and 150 cm. from the slit it is made to impinge on the end of a viewing box.

Here it passes through openings in an otherwise opaque cardboard. The shape of this cardboard is shown in Fig. 1. It is the pattern of a Lummer-Brodhun photometer made so as to be  $10 \times 10.5$  cm. Since the

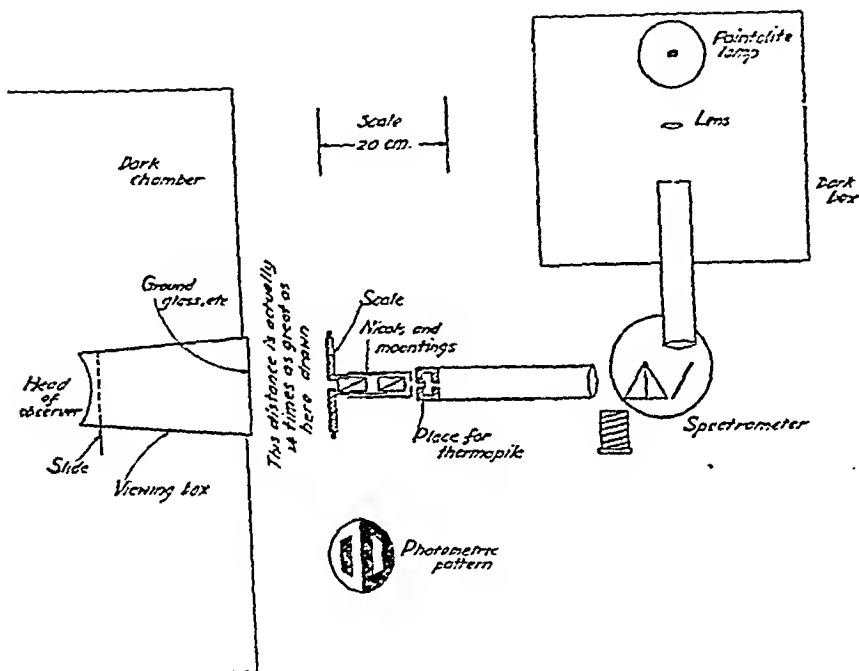


FIG. 1. Diagram of apparatus. The subject sits in the dark chamber curtained off from the rest of the dark room. Note that the front wall of this chamber is actually fourteen times as far from the spectrometer as shown in the drawing.

subject's eyes at the ocular end of the viewing box are 25 cm. from the pattern end, the pattern subtends a visual angle of  $22^\circ$ . The white portion of the pattern represents the parts cut out of the cardboard, through which the monochromatic light passes. In the viewing box and close up against this pattern are two sheets of ground glass which serve the double purpose of diffusing the light and reducing its intensity. The ground glasses are, however, so near the pattern that

though the light which comes through is diffused, it still possesses the shape given to it by the pattern.

This takes care of the variable monochromatic light. The constant comparison light is incorporated into the pattern of the viewing box. It consists of a layer of radium paint which covers the portion of the pattern represented as black in the figure. The layer of radium paint gives a constant and continuous illumination for long intervals of time (several years) provided it is not exposed to light. The brightness of this constant light was so adjusted as to be equivalent to an intensity of 2.7 times the threshold of visibility after complete dark adaptation. This was accomplished first by the two sheets of ground glass already referred to, and second by sheets of translucent paper cut into the proper shape and placed between the paint and the ground glass. The papers and the cardboard are held together by being mounted between two plates of ordinary glass.

The pattern end of the viewing box forms part of the wall of a dark chamber, curtained off from the rest of the dark room, in which the subject sits. In this way flashes of light used by the experimenter in taking readings and making adjustments are not seen by the subject, and hence do not spoil his dark adaptation. Moreover, a slide at the ocular end of the viewing box excludes even the experimental lights from the eyes of the subject between actual observations.

The intensity of the variable monochromatic beam is controlled by a coarse and a fine adjustment. The coarse adjustment consists of two neutral filters made of uniformly fogged photographic film, each reducing the intensity to  $\frac{1}{10}$ , and together to  $\frac{1}{100}$ . The fine adjustment between the range of the filters is made with the pair of Nicol prisms. The intensity which the nicols transmit is proportional to  $\cos^2\theta$ , where  $\theta$  is the angle between the prisms. At  $\theta=0^\circ$  the transmission is the maximum; at  $\theta=90^\circ$  the transmission is zero. The function  $\cos^2\theta$  varies slowly near  $0^\circ$ , and very rapidly near  $90^\circ$ . In order, therefore, to have a nearly constant rate of adjustment, the nicols were used between  $70^\circ$  and  $20^\circ$ . This is a range of approximately  $\frac{1}{10}$ , and thus covers the interval between the filters. By using the nicols always, first without any filter, then with Filter 1, and finally with Filters 1 and 2, it is possible to cover by a fine adjustment a range of intensities from 1 to  $\frac{1}{1,000}$  of the light transmitted by the nicols alone. This was more than ample for the experiments.

The position of the nicols is indicated in Fig. 1. The filters were placed directly in the path of the monochromatic beam immediately on its emergence from the slit of the spectrometer, in the groove built for the thermopile. The filters were mounted in wooden cases which were so arranged as to fit this groove, thereby insuring a constant and accurate position of the filter.

3. The apparatus as set up requires the calibration of the transmission of the filters, of the settings of the spectrometer, and of the energy distribution in the spectrum. The filters were calibrated photometrically three times, using two sources of light 2 meters apart, and a Lummer-Brodhun photometer. The results were practically the same each time. Filter 1 transmits 0.0977, and Filters 1 and 2 together transmit 0.00954 of the incident light.

The spectrometer, having once been set up, was not moved or readjusted during the experiments here recorded. It was calibrated<sup>1</sup> ten times during the investigation by means of the three hydrogen lines obtained from a hydrogen discharge tube. The results varied comparatively little from time to time, so that we may take their averages as indicating the correct values. In recording the wave-lengths, we use the values obtained graphically from the calibration curve made by plotting the actual values of the hydrogen lines against the drum readings.

The Pointolite lamp which we used runs on 230 volts and 4.5 amperes. We calibrated its energy content in the visible spectrum by means of a Hitchins thermopile and a Broca galvanometer. The resistance of the thermopile is 14.9 ohms, and that of the galvanometer 9.02 ohms. The results are shown in Fig. 2, in which each point is the average of four separate measurements. The energy content of the wave-lengths used in the experiments have been determined by graphic interpolation from this curve.

4. The method of taking observations is very simple, and requires no previous training or experience on the part of the subject. It merely requires him to be intelligent and interested. The subject is brought into the dark room, and 10 minutes are spent in explaining

<sup>1</sup> During the calibration of the spectrometer and of the energy content of the lamp, we had the help of Mr. R. A. Woodeson, to whom we wish to express our thanks.



the object of the experiment, the apparatus, and the procedure. He is then seated in the curtained off, dark chamber in front of the ocular end of the viewing box. Special precautions are taken to make the subject very comfortable, to have his back supported all the time,

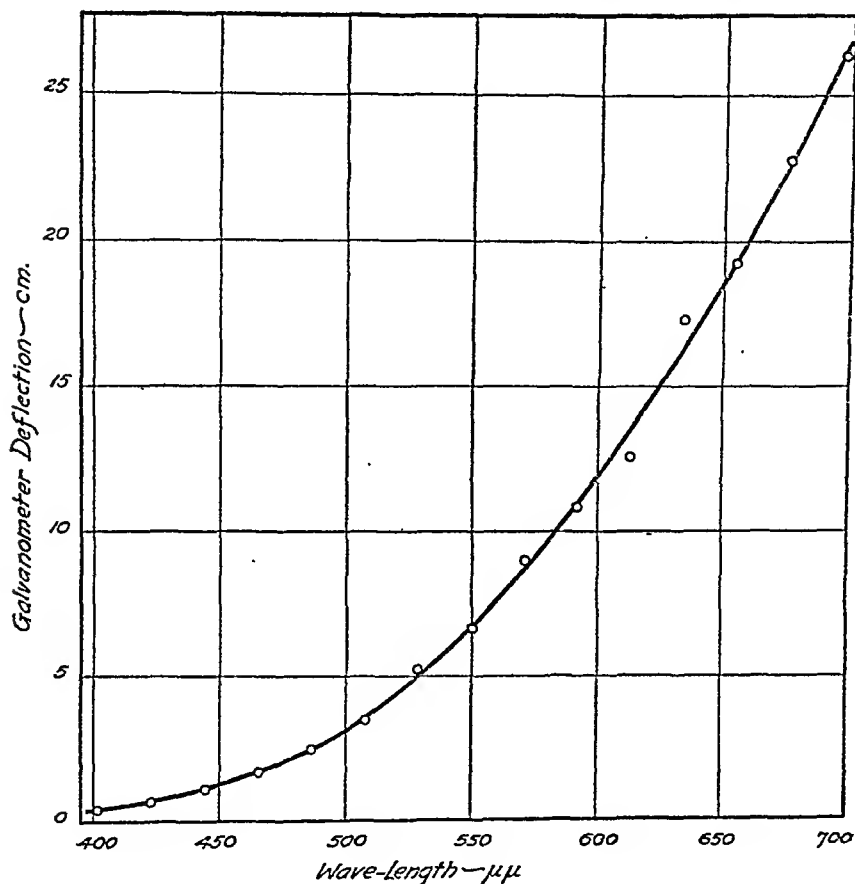


FIG. 2. Energy distribution in spectrum of a 500 candle power Pointolite lamp. Each point is the average of four determinations of the galvanometer deflection obtained from a thermopile placed in the position indicated in Fig. 1.

to have the chair the right height, etc., so that the act of sitting for an hour and a half or more will involve as little physical strain and discomfort as possible. The positions of the chair and the subject

are so adjusted that in order to look into the viewing box he is required to move his head forward only about 5 cm. We mention these details because we are convinced that the personal comfort of the subject has a decided influence on the smoothness and accuracy of the results.

The light from the spectrometer is cut off by placing the nicols at right angles, and the subject is allowed at least half an hour for dark adaptation. During this half hour the subject is kept interested by allowing him to watch the gradual appearance of the pattern formed by the radium paint, indicative of the increase in the sensitivity of his eyes. This pattern usually becomes faintly visible after 10 or 15 minutes. During the next 15 minutes it increases in brightness and clearness, so that the subject becomes familiar with its shape and learns how to find it when looking into the viewing box and pulling out the slide. The appearance of the pattern at this stage is that of an oval field of light with vertical dark bars in it representing the places where there is no radium paint and where the experimental light will come through later. The edges of the pattern are not sharp, because of the ground glass diffusion and the use of rod vision.

The subject is now given a few practise trials in the methods of making observations. The spectrometer is set for  $412\mu\mu$ , the nicols being still at right angles. The subject looks in and observes the shadows and the pattern. This takes a few seconds only; the slide is immediately replaced in the viewing box, and the subject remains in complete darkness. The movable nicol is then turned to admit some of the experimental light. The subject again observes the pattern and notices whether the vertical shadows are still as strong as they were before. More illumination is admitted, and the subject again examines the pattern. This is continued until the shadows have completely disappeared, and the field has become uniformly illuminated; the last observations are made slowly and in steps of about  $10'$  of arc. The final judgment of uniformity is always checked after a rest of a minute or so.

It should be emphasized that the errors and troubles of heterochromatic photometry do not exist here, because the field is uniform not only in lack of brightness contrast, but in lack of color as well.

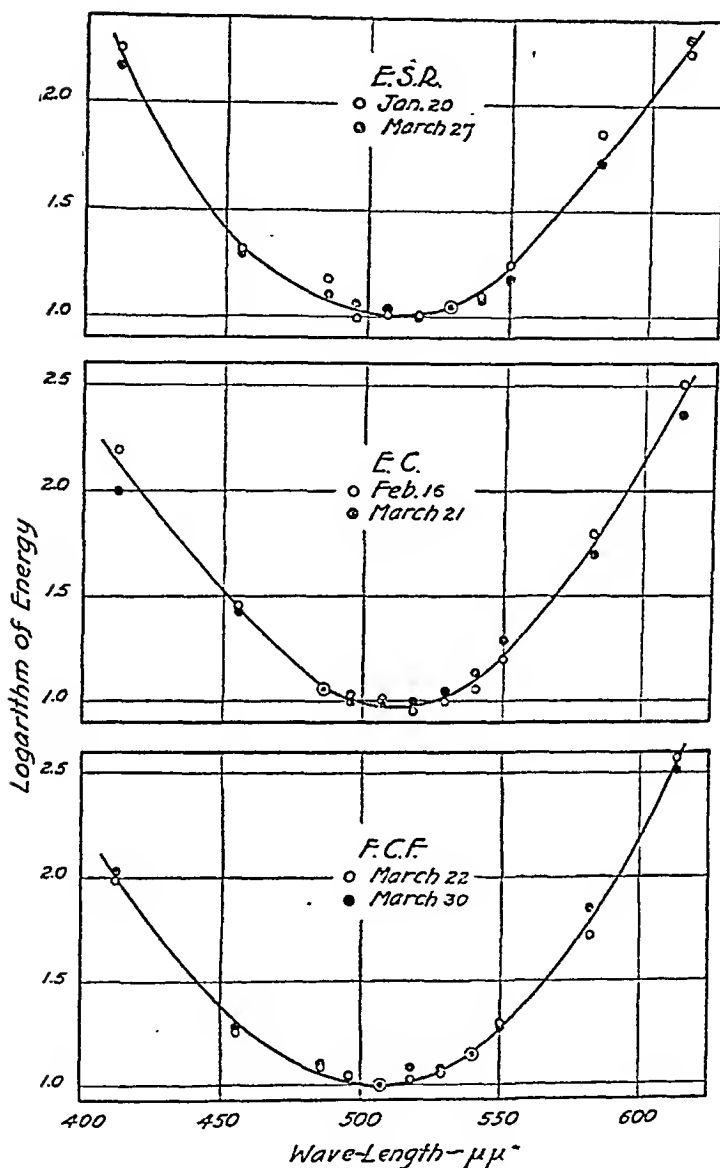


FIG. 3. Results with three subjects who came twice. The points are single readings. The results are reproducible for the same subject at different times, and are practically the same for the three subjects.

of this minimum value of 10. This served to get the data for all the subjects into a similar order of magnitude. The results for each wave-length for the 48 observations were then averaged. These averages are given in Table II, second column. In order to show them graphically, they are plotted logarithmically in Fig. 4.

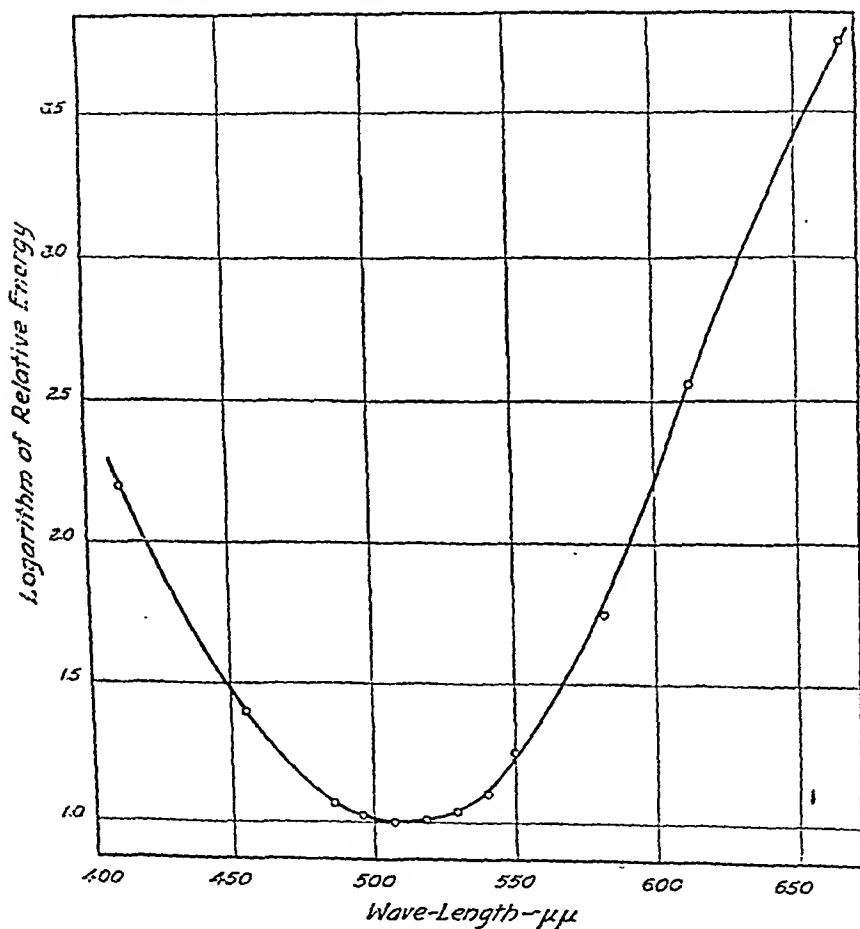


FIG. 4. Relation between energy for achromatic vision and wave-length. Each point is the average of 48 determinations.

It is apparent from Table II and Fig. 4 that the wave-length at which the minimum energy is necessary to produce a given brightness is very near 510  $\mu\mu$ . The energy values for the wave-lengths on both

sides of this point rise quickly, and in the orange and the violet are more than a thousand per cent greater than at the minimum.

Both the position of this minimum and the shape of the curve relating energy for vision with wave-length are of significance for an understanding of the photochemistry of visual reception. We shall therefore devote the remainder of this paper to these two points and to certain other matters which arise in connection with them.

TABLE II.

*Relation between Wave-Length and Energy for Vision and Visibility.*

Wave-length.	Relative energy.	Visibility.
$\mu\mu$		
412	158.1	6.32
455	25.03	39.95
486	11.99	83.40
496	10.65	93.90
507	10.06	99.35
518	10.28	97.30
529	10.98	91.10
540	12.69	78.78
550	17.99	55.60
582	56.24	17.78
613	367.2	2.72
666	5,525	0.181

## IV.

*Shape of Visibility Curve.*

It goes almost without saying that the rods must possess a photo-sensitive substance which has an absorption band in the visible spectrum. The effect of light on this substance is the initial event in its reception by the retina. Let us assume that in order to produce a certain sensation of gray in the eye the same amount of photochemical action must be induced in the sensitive substance, irrespective of the wave-length of the light. In terms of Grotthus' law, and its quantitative demonstration by the work of Lasareff (1907), this means that to produce the same visual effect the sensitive substance will have to absorb the same amount of energy regardless of the wave-length of the incident light.

Let us call this amount of energy  $E$ . At the position of maximum absorption  $\lambda_{max}$  of the sensitive substance,  $\frac{E}{a_{max}}$  units of incident light will be sufficient to yield that amount of absorbed energy,  $a_{max}$  being the absorption coefficient at the maximum. At any other wave-length  $\lambda$  the absorption coefficient  $a_\lambda$  will be less than at  $\lambda_{max}$ , but the amount of light necessary so that  $E$  units are absorbed will always be  $\frac{E}{a_\lambda}$ . In short, the amount of light  $A_\lambda$  necessary to produce a given sensation at any wave-length  $\lambda$  will be given by the equation

$$A_\lambda = k \frac{E}{a_\lambda}$$

By solving this equation for  $a_\lambda$  we get

$$a_\lambda = k \frac{E}{A_\lambda}$$

which gives us the value of the coefficient of absorption of the sensitive substance for any wave-length. The term  $k$  is a constant, and  $E$  is a constant; therefore the absorption coefficient of the sensitive substance for any wave-length is proportional to the reciprocal of the amount of energy necessary to produce a given sensation of gray at that wave-length.

It should then follow that the reciprocals of our data in Table II, second column, should represent the absorption spectrum of the sensitive substance in the condition in which it is in the retina. The continuous (low intensity) curve in Fig. 5 shows the shape taken by the data when plotted in this manner. The values of the points are taken from the third column of Table II, and are the reciprocals of those in Column 2 arranged so that the maximum of the curve has a value of 100. It is at once clear that the shape of the curve corresponds to the shape of the absorption bands of numerous well known substances.

It would be highly desirable to demonstrate this exact correspondence by applying an equation for the shape of an absorption band to the results in Fig. 5. Unfortunately this cannot be done with any great assurance in the present stage of our knowledge of absorption

spectra, because there exists no generally applicable equation for the shape of an absorption curve of a complicated substance in solution. In fact there exists no accepted interpretation of the shape of the absorption spectrum even of simple gases.

Several attempts have indeed been made to devise some formula for the shape of visibility curves (as the curves in Fig. 5 have been called) for high intensities (Nutting, 1908) as well as for low intensities

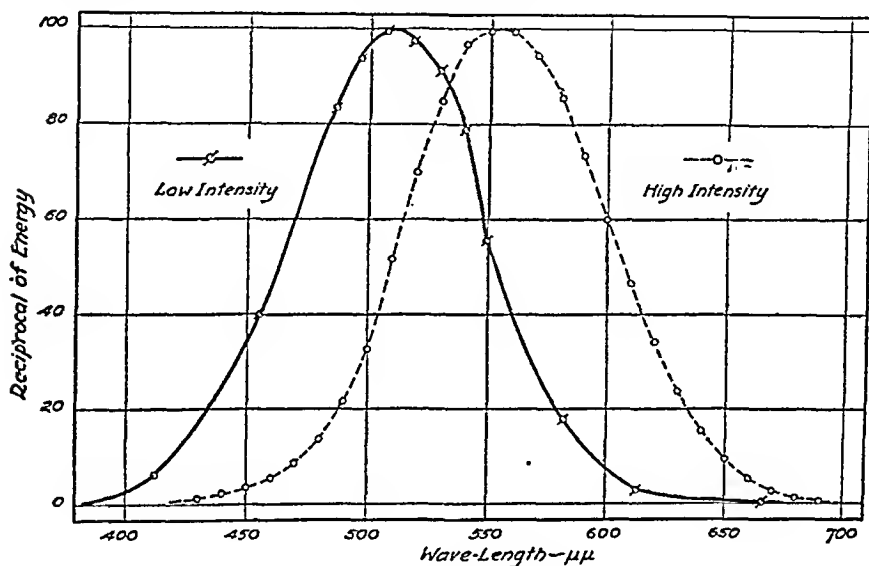


FIG. 5. Visibility curves for achromatic and chromatic vision. The ordinates are proportional to the absorption coefficients of the sensitive substances in the retina. The low intensity curve is therefore the absorption spectrum of the sensitive substance in the rods, while the high intensity curve is the absorption spectrum of the sensitive substance in the cones.

(Goldhammer, 1905; Renqvist, 1920). The best that may be said for these attempts is that they are amusing. They contribute little to the theoretical aspects of the matter, because they are all strictly empirical. Nutting's formula is based on a probability function, whereas Goldhammer's equation has not even that basis. Although nominally derived from Wien's formula for black body radiation, it is really an arbitrary relation between pure numbers possessing no dimensions, and as such it has not a trace of theoretical significance.

Rengqvist has tried to apply Planck's resonance theory of light absorption in gases to a calculation of Trendelenburg's data for visibility at low intensities. Aside from the fact that Trendelenburg's data are valueless for this purpose because he took account neither of the adaptation of his eye nor of the energy distribution in the gas flame which he used, the application of Planck's equations in this connection is rather arbitrary and is far from possessing any general significance.

Nutting's attempt to use the probability function is commendable, though he does not seem to recognise the significance of visibility curves in terms of absorption spectra. The symmetrical or nearly symmetrical curves that represent simple absorption spectra resemble strikingly the distribution curves of errors, of populations, etc. which are familiar in the theory of statistics. It may be that the shape of an absorption curve represents the fact that the absorbing substance is composed not of a uniform series of resonators, but of a group of resonators whose free periods may be expressed by the common distribution curves of the statisticians.

It is, however, not in our province to proceed any further along this line of reasoning. For our purposes it will suffice to note the similarity of our curve with curves showing the shape of absorption spectra, leaving aside for the moment the exact theoretical interpretation of such curves. We may then proceed to a comparison of our results with those obtained in the study of the brightness values of spectral light at high intensities in which color enters as a factor.

## V.

### *Chromatic and Achromatic Vision.*

In recent years many careful measurements have been made of the relative brightness of different parts of the spectrum at high intensities by methods of heterochromic photometry. The results of all the investigators have been about the same, except, as Hyde, Forsythe and Cady (1918) point out, that the curves obtained with the flicker photometer have in general been somewhat wider than those obtained by methods of direct photometry. The method which we used in our own data on achromatic vision is one of direct photometric



comparison. Without therefore entering into a discussion of the relative merits of the two methods of heterochromic photometry, we have chosen for comparison with our own, those data, which like our own, have been made with a method of direct photometry.

It has never been possible to make this comparison before except in the roughest manner (*cf.* Nutting), because the data for achromatic vision was so inadequate. Now that the necessary data have been made available, we may compare them with the recently reported measurements of Hyde, Forsythe and Cady with 29 subjects. These authors give their data as the reciprocals of the relative energy necessary to produce a certain brightness using different spectral colors. The curve which they present is reproduced in the broken (high intensity) curve of Fig. 5. It is at once clear that the curve also resembles the absorption spectrum of a substance having a band in the visible.

Even a superficial comparison of the two curves in Fig. 5 shows their similarity. To make an accurate demonstration of this resemblance we have proceeded as follows: We have measured the horizontal distances between the two curves in twelve places. The average of these twelve measurements turns out to be very nearly  $48 \mu\mu$ . We have then moved the curve of Hyde, Forsythe and Cady  $48 \mu\mu$  to the left, and have measured graphically the ordinates on their curve which correspond to the wave-lengths of the points on our curve. The two sets of points, theirs and ours, are plotted in Fig. 6. Their identity is shown by the fact that the same smooth curve passes through both sets of points. The agreement between the two sets of data is much better than the coincidence between the five or six visibility curves at high intensity which have already appeared from the careful work of different laboratories (*cf.* for example, the set of curves summarized in the paper by Hyde, Forsythe and Cady). It is therefore apparent that the two curves—one at high intensities involving color vision, and the other at low intensities involving only gray vision—are really the same curve in two positions on the spectrum  $48 \mu\mu$  apart.

In order to make the comparison absolutely accurate, the data at high intensities should be corrected for the absorption of the macula lutea. The values for the high intensities are measured with the

fovea, whereas those at low intensities are measured with the periphery. To compare the two results as far as their receptive elements are concerned, the macular absorption should be considered. Unfortunately, however, no decent data are available for this correction. Sachs (1891) measured the absorption of the yellow spot using a double collimator spectrophotometer of the Vierordt type. The results he gives are few and irregular, and it would be an insult to the beautiful

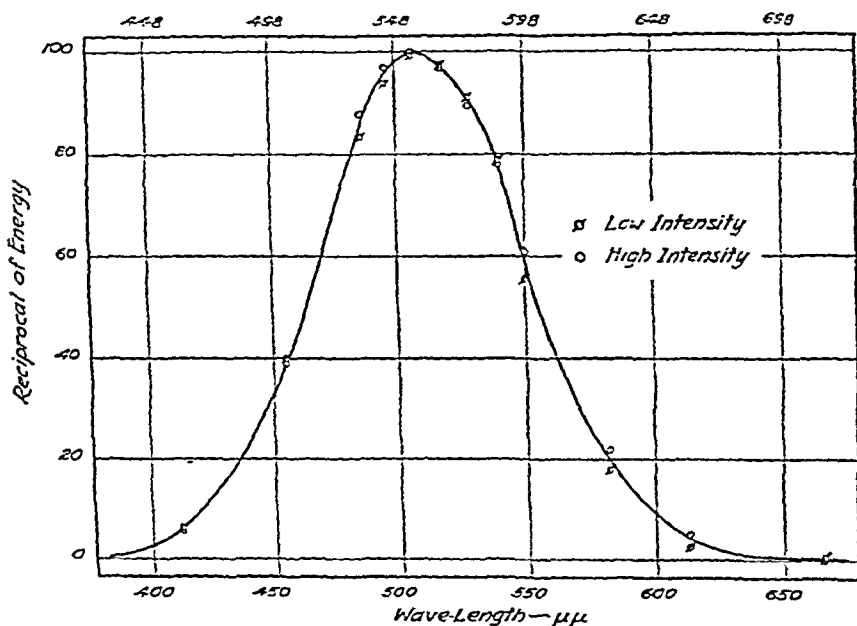


FIG. 6. Identity of high and low intensity curves of Fig. 5. The high intensity curve has been moved 48  $\mu\mu$  to the left, but its scale of abscissæ is given at the top of the figure.

data of Hyde, Forsythe, and Cady to subject them to a correction in terms of the rough and unreliable measurements of Sachs.

Nevertheless we have made this correction for our own interest, simply to see what influence it has on the results. It changes the shape of the curve slightly so as to make it more symmetrical, and it moves the maximum from 554 to 540  $\mu\mu$ . That macular absorption really accounts for this difference in the position of the maximum is demonstrated by the fact that visibility curves for colored lights

made with the fovea and with a region just outside of the macular area differ in the same direction and order of magnitude (Abney and Festing). The corrected curve still coincides with our points, but the difference between the two becomes only 39 instead of 48  $\mu$ . Though this reduction is favorable to our interpretation, it possesses no theoretical significance, and leaves the fundamental fact secure that the two brightness curves—one for chromatic vision and the other for achromatic vision—are essentially the same curves in different positions on the spectrum.

## VI.

### *Interpretation of Similarity of Visibility Curves.*

1. There is one implication that follows at once from this identity of the shape of the two curves. No one has ever objected to the assumption that a *single* substance is concerned with the reception of stimuli which produce achromatic effects at low intensities. If our reasoning in an early part of this paper is correct, the low intensity curve in Fig. 5 represents the absorption spectrum of this substance in the condition in which it is in the retina. In the visibility of color at high intensities we find a curve which has exactly the same shape and size as that for no color at low intensities. It would therefore seem the most obvious thing to assume that in the brightness perception of color, we are dealing also with the photochemical properties of a *single* photosensitive substance. As far as we ourselves are concerned, we definitely accept this assumption of a single photoreceptive substance as the necessary conclusion from the above comparison.

Precisely what this involves one cannot say. Many questions arise at once. For example, is this single substance the receptive agent for only the brightness values of colors, or is it to be regarded as the receptive material for color vision as a whole? There is no definite answer to this question at present. A certain amount of suggestive data exists for supposing that the brightness function may be distinct from the chromatic function, and the reader is referred to an extended discussion of the possibilities of this separation in a recent review by Troland (1922). The similarity of the brightness curve to the absorption spectrum of a single substance might be taken as additional

evidence for such a separation if one adopts a three or four substance explanation of color vision. To us, however, this is not a necessary step. The field of retinal physiology has hardly been touched from the viewpoint of modern photochemistry; and in view of the meagreness of our quantitative knowledge of the retina and of the photo-sensory behavior of dyes, it would be premature to decide the matter. All that we wish to conclude at present is that judged solely by the visibility curves, there is as much evidence for assuming only one receptive substance for chromatic vision as there has been for achromatic vision. There the matter must rest until further quantitative information becomes available.

2. There are certain points with regard to this photoreceptive substance, however, a consideration of which will lead to a clarification of our ideas. We can usually reason about the color of a substance from its absorption spectrum. The substance represented by the high intensity curve in Fig. 5 should, like the one represented by the low intensity curve, be purple, but should also be slightly more violet because its maximum is farther toward the red. This substance is to be found in the cones of the retina.

It will be at once objected that no such colored substance, in fact, no colored substance, has ever been demonstrated in the cones. The objection carries its answer with it. There are limits to the visibility of colored solutions. Make them sufficiently dilute, and look at them in thin layers, and they fail to be perceptible. The terminal segments of the cones, where presumably this substance is localized, form an extremely thin layer. If in addition the concentration of the substance is low, the result will be an apparently colorless medium. One who is inclined to be skeptical on this point need only look at a thin layer of a not too dilute solution of hemoglobin, or at a crystal of hemoglobin under the microscope, to realise how limited is our capacity in this respect.

The objection will then be raised that when the solution is so dilute, and the layer so thin, the photochemical characteristics of the substance will have reached their vanishing point. Weigert (1920) has recently given the counter to such an objection by his beautiful work on the photochemical properties of thin layers of  $\text{AgCl}$ , and of dyes like cyanin. Far from losing their photochemical characteristics,

these dilute, thin layers of material apparently acquire a number of new ones, which, as Weigert has pointed out (1921), will be of significance in any consideration of the mechanism of color vision.

We may therefore accept the proposition that the high intensity curve in Fig. 5 represents the absorption spectrum of a dilute solution of a photosensitive substance in the condition in which it is in the cones. This substance is the receptive material concerned with the production of such quantitative relationships as given by the visibility curve. The recent work of Weigert has given us but a hint of the possibilities for a mechanism to accomplish such effects. But we must not jump, like the many lost souls already on record, into the realms of visual theory.

3. We have then apparently two photosensitive substances in the retina. One is in the rods, and has its maximum of absorption at  $511 \mu\mu$ , while the other is in the cones, and has its absorption maximum at  $554 \mu\mu$  (or at  $540 \mu\mu$ , if we adopt the correction for the absorption of the macula). There are three logical possibilities for the relation between these two substances. They may be totally different substances; they may be similar substances differing perhaps in the position of the color-bearing groups in the molecule; and they may be identical.

The first possibility of complete diversity of structural relationship is made doubtful by the striking resemblance of the two absorption curves. Judging by the persistence of such substances as hemoglobin and chlorophyll in different groups of organisms, it might be argued that the organism would not produce two widely different substances located in the same sense organ and fulfilling very nearly the same function of brightness evaluation. The argument is admittedly weak; its significance lies in the plausibility of the other possibilities. Between the second and third possibilities there is little choice. The existence of two closely related substances would satisfy the situation, and there is something to be said for such an idea, as we shall presently see. We wish, however, to call attention to the possibility of complete identity.

The main point is the difference of  $48 \mu\mu$  (or of  $39 \mu\mu$  really) between the position of the two maxima. Such differences are, however, well known in spectroscopy, and we may be pardoned for calling attention

to them here. It had been known a long time that the same substance shows different positions for its absorption bands in different solvents; but it was Kundt (1878) who made the first systematic investigation of this phenomenon. Kundt wished to correlate the degree of refraction or of dispersion of a solvent with its effect on the position of the absorption bands of a substance dissolved in it. He was only partially successful, and the rule which bears his name, though having a number of exceptions, has been shown to hold true for many substances. In its most general form, Kundt's rule may be stated as follows. If one colorless solvent has a decidedly greater refracting or dispersing capacity than a second, then the absorption bands of a substance dissolved in the first will be nearer the red end of the spectrum than when dissolved in the second (Kayser, 1905).

Kundt's rule has had a particular field of support in the sensitizing properties of certain dyes on the photographic plate. Vogel (1874) found that the maximum of sensitivity of a photographic plate does not correspond with the maximum absorption of the added dye. The maximum of sensitivity is distinctly shifted toward the red. This work was later extended by Eder (1885) who showed that the shift is due to the actual position of the absorption maximum of the dye in the condition in which it is dissolved in the silver bromide grains. Eder obtained shifts toward the red varying from 15  $\mu\mu$  to as high as 65  $\mu\mu$ , while shifts to the extent of 31, 47, and 48  $\mu\mu$  were fairly common. Eder has pointed out that this corresponds to Kundt's rule because the density of the AgBr grains is 6.5 as compared with 1.0 for water and 1.3 for dried gelatin. The still more recent work of Sheppard (1909) has amply confirmed the findings of Vogel and of Eder.

The matter may be illustrated beautifully in the case of cyanin. Cyanin has a band in the red—hence its blue color. Dissolved in a film of collodion which is still moist, cyanin is blue. If the film is allowed to dry, thereby increasing the density and refractive index of the collodion, the film becomes colorless, because the absorption band has been shifted into the infra-red (*cf.* Weigert, 1921).

Enough has already been said to show how the same substance could give the two curves in Fig. 5, 48  $\mu\mu$  apart, depending on the refractive index or density of the medium in which it is dissolved.

The Duplicity theory makes a sharp distinction between colored and colorless vision, the one being located in the cones, and the other in the rods. It is conceivable that the terminal segment of the cones is much denser and more refractive than that of the rods, and therefore that the same substance dissolved in the cones would have its absorption band shifted strongly toward the red in comparison to its position in the rods.

This matter, however, is not one for speculation, but can be put to experimental test; we are already engaged in preparing the experiments. There is, nevertheless, a test which may not be without significance, and for which the data are already available. To this we shall now turn.

## VII.

### *Achromatic Brightness and Visual Purple.*

1. We have so far not mentioned the relationship of these phenomena to visual purple, because we have wished to consider the matter in some detail and from a point of view somewhat different than the one which has usually been employed (Bayliss, 1918). In 1894 Koenig measured the absorption spectrum of visual purple, and compared it with the brightness value of the spectrum to a color-blind person and to color-sensitive people at low intensities. He concluded that the two sets of curves were coincident, and deduced therefrom the idea that visual purple is the receptor substance for achromatic vision. Trendelenburg in 1904 next measured the rate of bleaching of visual purple in different parts of the spectrum. After measuring in addition his own visibility curve at low intensities, he compared the bleaching rates with his and Schatarnikoff's visibility curves, and reached a conclusion similar to that of Koenig. Later (1911) Henri and Languier des Bancel, without adding any experiments, recomputed the data of Koenig and Trendelenburg and compared them with the results of Schatarnikoff and Pflüger on the visibility of the spectrum at low intensities. The conclusion they reached was that in order to produce a visual sensation, the incident light must be of such intensity that the visual purple in the rods will always absorb the same energy regardless of wave-length. This, as the authors point out, is the conclusion of Koenig and of Trendelenburg in a slightly different form.

It will be noticed that these conclusions all depend on the coincidence of the curve of the absorption spectrum of visual purple with that of the curve for the visibility of the spectrum at low intensities. We have already pointed out at the beginning of this paper how unreliable are the data describing the visibility of the spectrum at low intensities. Indeed it was to supply this deficiency that our experiments were made. Now that we have secured these accurate data, it will be interesting to compare them with those of the absorption of visual purple in order to see whether this much discussed coincidence is real or only the product of insufficient data.

Before we do that, however, we wish first to say that, though we accept the conclusions of Koenig, Trendelenburg, and Henri and Larguier des Bancelles with regard to the relation of visual purple and achromatic vision, we cannot accept the evidence on which they are based. In fact, we believe that their evidence actually proves something quite different from what they thought. This paradox deserves an explanation.

2. The terminal segments of the rods are, from all observations and descriptions, fairly dense and highly refractive bodies—much more so, for example, than water (von Helmholtz, 1909). It is in these that the visual purple is dissolved in the living retina. If we may argue from Kundt's rule, which we explained in the previous section, the position of the absorption maximum of visual purple in the rods will not be the same as that in solution in water or in dilute bile salts solution. The absorption band of the visual purple in the rods should be shifted toward the red because the medium is much denser and more refractive than bile salts solution. The sensitivity of the rods to spectral light should, therefore, just as the sensitivity of a stained photographic plate, be farther toward the red than would be expected from the position of the absorption spectrum of visual purple as measured in bile salts solution.

If this were found to be true, not only would it strengthen our reasoning in this matter, but it would lend support to the possible explanation which we ventured for the difference in position of the maximum of sensitivity of the rods and the cones. Indeed, it was the idea of this explanation that first led us to examine the data from this critical point of view.



3. The absorption spectrum of visual purple has been measured on three occasions. The first was by Koenig in 1894. Koenig had built a new modification of the Vierordt spectrophotometer for just this work. But before he could acquire any technic with visual purple he came into the possession of a single human retina from a freshly extirpated eye. The temptation was too great, and even though the solution which he prepared was cloudy, he and Köttgen measured its absorption spectrum. The extended and fantastic theories which Koenig developed as a result of his measurements with this single retina are open for him to read who will. It is not for us to dwell too harshly on these lapses. If a datum is worth publishing it is worth interpreting to the limits of its capacity; and the criteria for publication are largely matters of taste, which are not in the realm of discussion. It will suffice to say that, though Koenig's curve for the absorption spectrum is none too regular, it is sufficiently so to show clearly that its maximum is very near  $500 \mu\mu$ .

After more practise with the new spectrophotometer and with the technic of preparing visual purple solutions, Köttgen and Abelsdorff (1896) took up the study of the absorption spectrum of visual purple in earnest. They made a large number of measurements with visual purple from a variety of animals, and their results are indicative of careful work. They found that visual purple prepared from the monkey, the cat, the rabbit, and the frog, gives the same absorption spectrum. We have studied their individual measurements in great detail and in every instance for every preparation the maximum of absorption is the same; that is, very near  $500 \mu\mu$ . The individual readings between 620 and  $460 \mu\mu$  agree very well with one another, and may be accepted with confidence, short of course, of an error in the calibration of the prism. However, the last two readings in the red (660 and  $640 \mu\mu$ ) and in the violet (420 and  $440 \mu\mu$ ) are subject apparently to a large experimental error, because the individual values vary as much as 100 per cent from one another, besides occasionally yielding the theoretically impossible negative value for the absorption coefficient. We have therefore not included these four points in a consideration of their data.

The third determination of the absorption of visual purple was made by Trendelenburg (1904). He made no systematic measure-

ments, but on six occasions during his work on the bleaching of visual purple, he measured the absorption spectrum over short intervals now here, now there. The concentrations were never the same, and after making proper corrections, he averaged these irregular results. He then plotted them in comparison with those obtained by Köttgen and Abelsdorff. Though it is obvious that his curve lies consistently  $5\ \mu$  farther toward the red, he makes no comment on the comparison. We fail to understand this difference, and must attribute it to the irregularity of the measurements; but we are surprised that the disagreement was allowed to pass without comment.

Of the three sets of measurements, those of Köttgen and Abelsdorff are therefore the only ones that can be considered as fulfilling the criteria of accuracy. Although all three sets of measurements show, without question, that the maximum of the spectrum visibility is distinctly farther toward the red than the absorption maximum of visual purple in bile salts solution, we can make reliable comparisons only with those of Köttgen and Abelsdorff. These are presented in Fig. 7. The solid curve with the small circles is the visibility curve at low intensity taken directly from Fig. 5., and according to our reasoning represents the absorption spectrum of visual purple in the condition in which it is in the terminal segments of the rods. The other points and the broken line are the data of Köttgen and Abelsdorff for the monkey and the rabbit, their original values having been recomputed so as to give the maximum a value of 100. Far from the two curves being coincident, it is apparent that the absorption spectrum of visual purple in the eye is definitely and consistently farther toward the red than in water solution. The two curves are, however, identical, and represent no doubt the same substance in two different media obeying Kundt's rule.

4. This having been established, it will be instructive to examine more closely the curves presented by the various authors in support of the coincidence of the two sets of observations. Koenig's figure shows beyond question that not only the maximum but almost the entire curves for the brightness perception of the spectrum of a color-blind person and of color-sensitive people at low intensities are distinctly farther toward the red than the maximum and curve for the absorption of visual purple. We have carefully replotted on a larger

scale the data given by Koenig, and find that the average difference between the two sets of data is about  $5\mu$ .

We have already pointed out the discrepancy of Trendelenburg's absorption data. But even here, if one examines the curves in Fig. 7 of his paper, one sees that the *Dämmerungswerte* are distinctly shifted an average of  $8\mu$  farther toward the red than the calculated absorp-

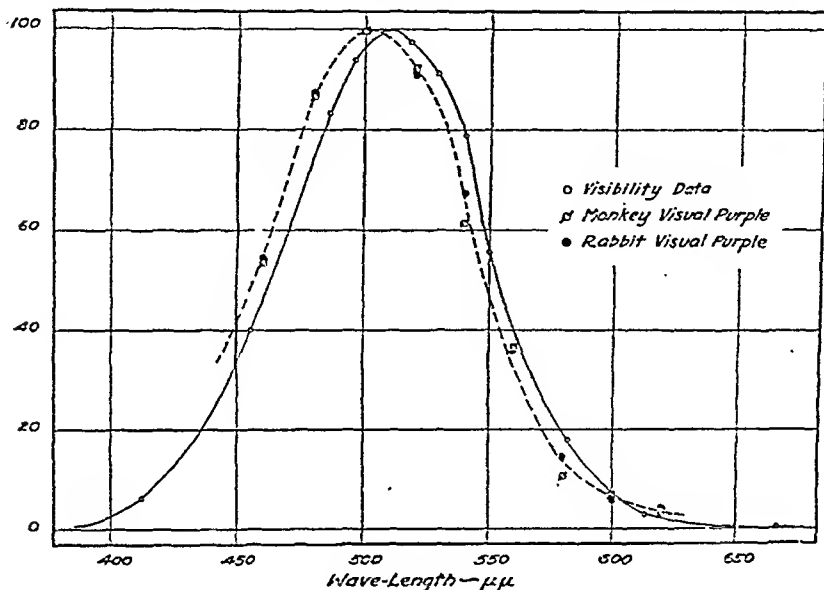


FIG. 7. Relation between absorption spectrum of visual purple in bile salts solution and absorption spectrum of sensitive substance in the rods as given by the visibility curve of Fig. 5. Though the two curves are identical, the visibility curve is shifted 7 or  $8\mu$  toward the red, as would be expected in terms of Kundt's rule.

tion of visual purple as determined by Trendelenburg. The main work of this author, however, is the bleaching effect of different wavelengths, which is probably quite accurate. If we compare the curve for the bleaching values with that for the dim values (*Dämmerungswerte*) of the same spectrum, we find here again that not only the maximum but the entire curve is displaced toward the red, an average distance of about 5 or  $6\mu$ .

Finally, in the paper by Henri and Languier des Bancelles, if we compare the curve representing the energy necessary to produce the same absorption in visual purple with the curve for the energy necessary to see the spectrum at low intensities, we find exactly the same shift of the latter toward the red. The curves are quite irregular, and it is hard to estimate the exact extent of the shift, but it varies between 5 and 20  $\mu\mu$  in different parts. Any one who cares may see this for himself in the reproduction of Henri and Languier des Bancelles' figure in Bayliss' book on General Physiology.

In all the cases then, the shift, whether large or small, is consistently present, and is always in the same direction, toward the red. Therefore, as we pointed out, though we believe the conclusion expressed by these authors, we find that the evidence they present fails utterly to prove it. More than that, their data actually prove something else, which is quite as significant as what they were supposed to prove. This is that the absorption spectrum of visual purple in the rods, following Kundt's rule, is definitely shifted toward the red for a distance of 7 or 8  $\mu\mu$ . It is to this shifted absorption spectrum that, we believe, the proportionality between visibility and absorption must be referred.

It is hardly necessary to indicate that this point too may be tested experimentally; but we do so in order to announce that we are preparing to perform the necessary experiments.

5. There is just one more point we wish to make about the absorption spectrum of visual purple and its relation to the visibility of radiation. We suggested the possibility that the two visibility curves of Fig. 5 are manifestations of the same sensitive substance, and that the difference in the position of the two curves is explainable in terms of Kundt's rule. This would assume that the visual purple which we can extract from the eye and whose maximum of absorption in bile salts solution is at 503  $\mu\mu$  is present in solution in the rods where its absorption maximum is at 511  $\mu\mu$ , and in very dilute solution in the cones where its maximum is at 554  $\mu\mu$  (or more probably, as corrected for macular transmission, at 540  $\mu\mu$ ).

The shift between the rods and the cones is a pretty big one. Not that such shifts are uncommon in the sensitization of a photographic plate, as Eder's figures amply testify; but that such a shift has to be

produced by a pretty big increase in density and refractive index. It may be that the terminal segments of the cones actually possess this greatly increased density or refractivity; that is something that will have to be settled experimentally. There is however another consideration.

We pointed out that one possibility in this relationship is that there may be two closely related forms of the sensitive substance differing slightly in some detail of molecular arrangement, which would account equally well for the shift in the absorption spectrum. There do exist what appear to be two such forms of visual purple. Köttgen and Abelsdorff, in their study of visual purple, found a sharp distinction between the substance obtained from fishes and from all other vertebrates. The maximum for the visual purple of all other vertebrates is, as we have given it above,  $503\text{ }\mu\mu$ . That from fishes is definitely more violet, and its absorption maximum is at  $540\text{ }\mu\mu$ . This difference is too consistent for it to be anything but a real one.

This is all that is known. Whether the fish form of visual purple is the one that is present in the cones, or what this fish modification of visual purple is, are questions that cannot even be speculated on at the present time. We even refrain from the tempting, but probably quite futile, discussion of the evolutionary aspects of the situation. It is clear, however, that the field is ready for experimentation, and we hope that we may be fortunate enough to find some solution for these stimulating and provoking questions which strike at the basis of the mechanism of vision.

#### SUMMARY.

1. After a consideration of the existing data and of the sources of error involved, an arrangement of apparatus, free from these errors, is described for measuring the relative energy necessary in different portions of the spectrum in order to produce a colorless sensation in the eye.

2. Following certain reasoning, it is shown that the reciprocal of this relative energy at any wave-length is proportional to the absorption coefficient of a sensitive substance in the eye. The absorption spectrum of this substance is then mapped out.

3. The curve representing the visibility of the spectrum at very low intensities has exactly the same shape as that for the visibility at high intensities involving color vision. The only difference between them is their position in the spectrum, that at high intensities being  $48 \mu\mu$  farther toward the red.

4. The possibility is considered that the sensitive substances responsible for the two visibility curves are identical, and reasons are developed for the failure to demonstrate optically the presence of a colored substance in the cones. The shift of the high intensity visibility curve toward the red is explained in terms of Kundt's rule for the progressive shift of the absorption maximum of a substance in solvents of increasing refractive index and density.

5. Assuming Kundt's rule, it is deduced that the absorption spectrum of visual purple as measured directly in water solution should *not* coincide with its position in the rods, because of the greater density and refractive index of the rods. It is then shown that, measured by the position of the visibility curve at low intensities, this shift toward the red actually occurs, and is about 7 or  $8 \mu\mu$  in extent. Examination of the older data consistently confirms this difference of position between the curves representing visibility at low intensities and those representing the absorption spectrum of visual purple in water solution.

6. It is therefore held as a possible hypothesis, capable of direct, experimental verification, that the same substance—visual purple—whose absorption maximum in water solution is at  $503 \mu\mu$ , is dissolved in the rods where its absorption maximum is at  $511 \mu\mu$ , and in the cones where its maximum is at  $554 \mu\mu$  (or at  $540 \mu\mu$ , if macular absorption is taken into account, as indeed it must be).

The experiments for this paper were performed in the George Holt Physics Laboratory of the University of Liverpool. We take this opportunity of thanking Professor L. R. Wilberforce for his kindness in placing the facilities of the laboratory at our disposal. It is a pleasure also to record our thanks to Professor E. C. C. Baly for the use of apparatus from the Laboratory of Inorganic Chemistry, and above all, for the generous hospitality of his laboratory.

## BIBLIOGRAPHY.

- Abney, W. deW., and Festing, E. R., Colour photometry. III, *Phil. Tr. Roy. Soc. London*, 1893, clxxxiii, A, 531.
- Bayliss, W. M., Principles of general physiology, 2nd edition, London, 1918.
- Ebert, H., Ueber den Einfluss der Schwellenwerthe der Lichtempfindung auf den Charakter der Spectra, *Ann. Physik. u. Chem.*, 1888, xxxiii, 136.
- Eder, J. M., Ueber die Wirkung verschiedener Farbstoffe auf das Verhalten des Bromsilbers gegen das Sonnenspectrum, (1885) reprinted in *Beitr. Photochem. u. Spectralanal.*, 3te Abt., 1904, 26.
- Goldhammer, D. A., Die Farbenempfindlichkeit des Auges und die photometrische Helligkeit der leuchtender Körper, *Ann. Physik.*, 1905, xvi, 621.
- Hecht, S., The dark adaptation of the human eye, *J. Gen. Physiol.*, 1919-20, ii, 499.
- von Helmholtz, H., Handbuch der Physiologischen Optik, Hamburg and Leipsic, 3rd edition, 1909, i.
- Henri, V. and Larguier des Bancel, J., Photochimie de la rétine, *J. physiol. et path. gén.*, 1911, xii, 841.
- Hillebrand, F., Über die specifische Helligkeit der Farben, *Sitzungsber. K. Akad. Wiss., Math.-Naturw. Cl.*, 3te Abt., 1890, xcvi, 70.
- Hyde, E. P., Forsythe, W. E., and Cady, F. E., The visibility of radiation, *Astrophys. J.*, 1918, xlviii, 65.
- Kayser, H., Handbuch der Spectroscopie, Leipsic, 1905, iii.
- Koenig, A., Ueber den menschlichen Sehpurpur und seine Bedeutung für das Sehen, *Sitzungsber. Akad. Wiss., Berlin*, 1894, 577; reprinted in *Ges. Abhandl.*, Leipsic, 1903.
- Köttgen, E., and Abelsdorff, G., Absorption und Zersetzung des Sehpurpurs bei den Wirbeltieren, *Z. Psychol. u. Physiol. Sinnesorg.*, 1896, xii, 161.
- Kundt, A., Ueber den Einfluss des Lösungsmittels auf die Absorptionsspectra gelöster absorbirender Medien, *Ann. Physik. u. Chem.*, 1878, iv, 34.
- von Kries, J., and Nagel, W., Über den Einfluss von Lichtstärke und Adaptation auf das Sehen des Dichromaten (Grünblinden), *Z. Psychol. u. Physiol. Sinnesorg.*, 1896, xii, 1.
- Langley, S. P., Energy and vision, *Am. J. Sc.*, 1888, xxxvi, 359.
- Lasareff, P., Über das Ausbleichen von Farbstoffen im sichtbaren Spectrum, *Ann. Physik.*, 1907, xxiv, 661.
- Nutting, P. G., The luminous equivalent of radiation, *Bull. U. S. Bureau of Standards*, No. 103, 1908, 261.
- Pfüger, A., Ueber die Farbenempfindlichkeit des Auges, *Ann. Physik.*, 1902, ix, 185.
- Renqvist, Y., Ein Versuch, die Plancksche Resonatoretheorie der Lichtabsorption auf die Absorption des Sehpurpurs anzuwenden, *Skand. Arch. Physiol.*, 1920, xl, 226.
- Sachs, M., Ueber die specifische Lichtabsorption des gelben Fleckes der Netzhaut, *Arch. ges. Physiol.*, 1891, i, 574.

- Schaternikoff, M., Neue Bestimmungen über die Vertheilung der Dämmerungswerthe im Dispersionsspectrum des Gas- und des Sonnenlichts, *Z. Psychol. u. Physiol. Sinnesorg.*, 1902, xxix, 255.
- Sheppard, S. E., The optical and sensitizing properties of the isocyanine dyes, *J. Chem. Soc.*, 1909, xcv, 15.
- Trendelenburg, W., Quantitative Untersuchungen über die Bleichung des Sehpurpurs in monochromatischem Licht, *Z. Psychol. u. Physiol. Sinnesorg.*, 1905, xxxvii, 1.
- Troland, L. T., Brilliance and chroma in relation to zone theories of vision, *J. Opt. Soc. Am.*, 1922, vi, 3.
- Vogel, H., Ueber die Beziehungen zwischen chemischer Wirkung des Sonnenspektrums, der Absorption und anomalen Dispersion, *Ber. chem. Ges.*, 1874, vii, 976.
- Weigert, F., Über einen neuen Effekt der Strahlung, *Z. Physik.*, 1920, iii, 437; Ein photochemisches Modell der Retina, *Arch. ges. Physiol.*, 1921, cxc, 177.





# THE COLLOIDAL BEHAVIOR OF SERUM GLOBULIN.

By DAVID I. HITCHCOCK.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 28, 1922.)

## I.

### INTRODUCTION.

The theory of the colloidal behavior of proteins developed by Loeb<sup>1</sup> has been shown to apply to the proteins, gelatin, casein, egg albumin, and edestin.<sup>2</sup> It may be recalled that this theory is based on the idea that proteins are amphoteric electrolytes, reacting stoichiometrically with acids and bases to form ionizable salts, and on Donnan's theory of membrane equilibrium.<sup>3</sup> The object of the present work was to find out whether the theory would explain the behavior of a serum globulin as well.

The globulin was prepared from serum which was obtained by whipping and centrifuging fresh ox blood. The serum was diluted and the globulin was precipitated near its isoelectric point by CO<sub>2</sub> and purified as described by Robertson.<sup>4</sup> The product was therefore the insoluble serum globulin or euglobulin. The isoelectric point of this globulin was found by Rona and Michaelis<sup>5</sup> to be at a hydrogen ion concentration of  $0.36 \times 10^{-5}$  (pH 5.44). 1 per cent suspensions of the present preparation in distilled water were found to have a pH of 5.41 or 5.42 at 33°C. The removal of salts was assured by a measurement of the conductivity of a 1.628 per cent suspension

<sup>1</sup> Loeb, J., *Proteins and the theory of colloidal behavior*, New York and London, 1922; *J. Gen. Physiol.*, 1918-22, i-iv.

<sup>2</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 597.

<sup>3</sup> Donnan, F. G., *Z. Elektrochem.*, 1911, xvii, 572.

<sup>4</sup> Robertson, T. B., *The physical chemistry of the proteins*, New York, London, Bombay, Calcutta, and Madras, 1918, 40.

<sup>5</sup> Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193. Michaelis, L., *Die Wasserstoffionenkonzentration*, Berlin, 1914, 56.

of the globulin in distilled water; the specific conductivity at  $34^{\circ}$  was found to be  $1.2 \times 10^{-5}$  reciprocal ohms. The globulin was kept in suspension in distilled water saturated with thymol, and was preserved in an ice box. To obtain the concentrations of globulin required for the experiments, the bottle was shaken and samples were withdrawn by a pipette. The accuracy of this method of measuring the globulin was checked by dry weight determinations on two 25 cc. samples; each was found to contain 0.407 gm. of dry globulin.

Inasmuch as this globulin preparation did not give clear solutions with either acid or alkali, it is probable that it had become partly denatured or changed in some way during the process of purification. A second lot was prepared which gave nearly clear solutions in acid or alkali. Nevertheless, the first preparation could still be used to show whether or not a protein prepared from ox serum obeyed the same laws as other proteins.

## II.

### *Titration of Globulin with Acids and Bases.*

Titration curves were obtained by measuring with the hydrogen electrode, at  $33^{\circ}$ , the pH of 1 per cent solutions of the globulin in HCl and  $H_3PO_4$  of various concentrations, and of a 0.5 per cent solution of the globulin in  $H_3PO_4$ . The pH values were referred to 0.1 M HCl, its pH being taken as 1.036. The results are given in Table I.

In order to determine how much of the HCl was combined with the globulin, the amounts of HCl required to give the same pH to 100 cc. of water, without protein, were subtracted from the total amounts of HCl in Table I. The figures for the acid-water curve have been given in a previous paper.<sup>6</sup> In the case of  $H_3PO_4$  it was pointed out in connection with the titration of edestin<sup>3</sup> that this method does not give the true amounts of combined acid, on account of the repression of the ionization of the weak acid  $H_3PO_4$  by the  $H_2PO_4^-$  ion from the protein phosphate. Accordingly the amounts

<sup>6</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, iv, 733.

TABLE I.  
*Titration of Globulin with Acids.*

0.1M HCl in 100 cc. of 1 per cent glo- bulin, cc.....	0	0	0.60	0.60	1.20	1.20	2.39	2.51	4.18	4.54	6.57	6.57	8.95	8.97	11.35	11.36	16.14	16.95	22.12	29.85	37.52	61.3
pH.....	5.41	5.42	3.99	4.07	3.71	3.80	3.43	3.39	3.16	3.07	2.85	2.86	2.60	2.62	2.35	2.36	2.04	2.08	1.85	1.67	1.52	1.29
0.1M $\text{H}_2\text{PO}_4$ in 100 cc. of 1 per cent globulin, cc.....	1.39	2.78	5.56	9.72	16.7	24.3	34.0	43.6														
pH.....	3.63	3.33	2.98	2.65	2.33	2.14	1.98	1.88														
0.1M $\text{H}_2\text{PO}_4$ in 100 cc. of 0.5 per cent globulin, cc.....	0.69	1.39	2.78	4.16	6.94	11.11	18.05	27.8	41.7	69.4												
pH.....	3.67	3.41	3.12	2.89	2.60	2.37	2.15	1.99	1.84	1.67												

of combined  $\text{H}_3\text{PO}_4$  were calculated by the equation<sup>7</sup> which was used in the case of edestin

$$x = \frac{kc}{h+k} - h$$

Here  $x$  = concentration of  $\text{H}_2\text{PO}_4^-$  from protein phosphate (assumed to be completely ionized).

$k$  = primary ionization constant of  $\text{H}_3\text{PO}_4 = 0.01$ .

$c$  = total concentration of  $\text{H}_3\text{PO}_4$ .

$h$  = concentration of  $\text{H}^+$  = concentration of  $\text{H}_2\text{PO}_4^-$  from  $\text{H}_3\text{PO}_4$ .

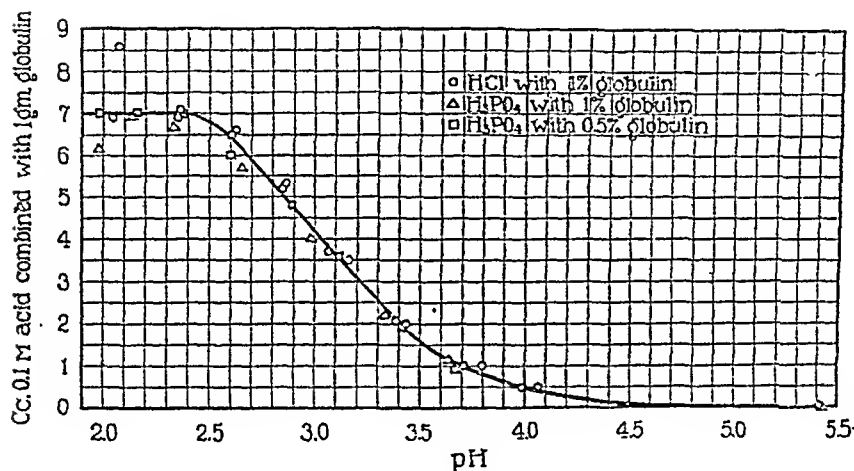


FIG. 1. Combination of globulin with acids.

The values obtained in this way, together with the values for HCl, are plotted in Fig. 1 in terms of cc. of 0.1 M acid combined with 1 gm. of globulin. Since the points fall on one curve it is to be inferred

<sup>7</sup>An equation identical with this was used by Pauli and Hirschfeld (Pauli, W., and Hirschfeld, M., *Biochem. Z.*, 1914, lxii, 245; Pauli, W., *Kolloidchemie der Eiweisskörper*, Dresden and Leipsic, 1920, pt. 1, 57) to calculate the amounts of acetic acid combined with horse serum albumin. However, since they plotted the amounts of combined acid against the total concentration of acid added instead of against the pH, they were not able to show that the protein was combined with chemically equivalent amounts of weak and strong acids.

that the globulin reacts stoichiometrically with equal numbers of molecules of the two acids. In other words, both  $\text{HCl}$  and  $\text{H}_3\text{PO}_4$  react with globulin as monobasic acids. The combination curve appears to become horizontal at about 7 cc. of 0.1  $\text{N}$  acid, indicating a combining weight of about 1,400 for the globulin. However, the height of the maximum is more or less uncertain. In attempting to carry the curve into the region of lower pH, it was found that the points for  $\text{HCl}$  varied irregularly on both sides of the value 7 cc.,

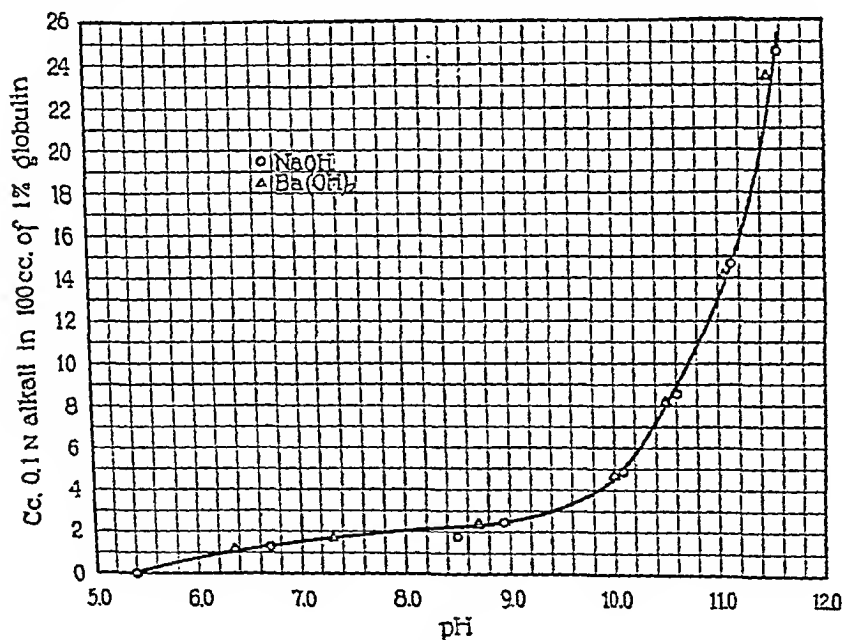


FIG. 2. Titration of globulin with alkalis.

while those for  $\text{H}_3\text{PO}_4$  seemed to descend toward the axis of abscissæ. The former deviations are evidently due to the magnification of errors involved in taking the differences between two steep curves; the apparent decrease in the amount of  $\text{H}_3\text{PO}_4$  combined is probably due to incomplete ionization of the globulin phosphate in the presence of much  $\text{H}_3\text{PO}_4$ , while in the calculation it was assumed that the protein phosphate was completely ionized. At any rate the results in Fig. 1 show that between pH 2 and 4 the globulin reacts stoichiometrically with equimolecular amounts of the two acids.

It should be added that a potentiometric titration of serum globulin with HCl was made by Robertson.<sup>8</sup> He calculated the amount of combined HCl simply by taking the difference between the total acid concentration present and the hydrogen ion concentration found in the presence of the protein. This involves the assumption of complete ionization of the HCl, which is probably correct for the concentrations he used, 0.01 M or below. His values fall at points higher than those in Fig. 1, and do not appear to reach a maximum in the range of acidity which they cover. The cause for this difference may lie in differences in the globulin preparations, or possibly in differences in temperature or in the standards of hydrogen ion concentration.

Fig. 2 represents the titration of 1 per cent globulin with NaOH and Ba(OH)<sub>2</sub>. The curve is the original titration curve; no attempt was made to calculate the amount of combined alkali. These two strong alkalis appear to give the same titration curve with globulin when the concentrations are plotted in terms of normality, indicating that the globulin combines with them in equivalent, not in molecular proportions.

These titration experiments indicate that serum globulin behaves like gelatin, casein, egg albumin,<sup>1</sup> and edestin<sup>2</sup> in its stoichiometric reactions with acids and bases.

### III.

#### *Membrane Potentials.*

In order to determine whether the Donnan equilibrium applied to the behavior of serum globulin, experiments were carried out by the method which had been used by Loeb<sup>1</sup> with gelatin and egg albumin and followed by the present writer<sup>2</sup> with edestin. Solutions were prepared containing 1 gm. of globulin in 100 cc. of HCl of various concentrations. These were placed in 50 cc. collodion bags fitted with rubber stoppers and manometer tubes, and the bags were suspended in beakers of HCl free from protein but of about the same pH as the protein solutions. The beakers were placed in a water bath at  $25^{\circ} \pm 1^{\circ}\text{C.}$ , and about 20 to 24 hours were allowed for the

<sup>8</sup> Robertson, T. B., *J. Phys. Chem.*, 1907, xi, 437; The physical chemistry of the proteins, New York, London, Bombay, Calcutta, and Madras, 1918, 99.

attainment of equilibrium. Then the osmotic pressure was measured in terms of millimeters of the solution in the manometer tubes, and the p.d. between the inside and outside solutions was measured with the aid of saturated KCl-calomel electrodes and a Compton electrometer. The p.d. measurements were made at about 25°. The pH of the inside and outside solutions, at 33°, was then measured with the hydrogen electrode and potentiometer. The calculated p.d. values

TABLE II.

*Effect of pH on p.d. and Osmotic Pressure of 1 Per Cent Globulin Chloride.*

pH inside.....	4.57	4.38	3.86	3.55	3.35	3.06	2.67	2.19	1.72	1.28
pH outside.....	4.27	4.12	3.54	3.21	3.02	2.75	2.44	2.07	1.67	1.28
Observed p.d., millivolts ..	6.5	7.0	16.5	21.0	21.0	20.0	15.0	10.5	4.0	3.0
Calculated p.d., millivolts ..	17.5	15.0	19.0	20.0	20.0	18.0	13.5	7.5	3.0	0
Osmotic pressure, mm. ...	9	12	23	38	57	73	71	48	27	45

*Effect of pH on p.d. and Osmotic Pressure of 0.5 Per Cent Globulin Phosphate.*

pH inside.....	3.57	3.26	2.95	2.76	2.50	2.30	2.09	1.97	1.83	1.66
pH outside.....	3.32	3.00	2.70	2.54	2.35	2.19	2.02	1.90	1.78	1.64
Observed p.d., millivolts ..	12.0	13.0	13.0	11.5	9.0	7.0	5.0	4.0	3.5	2.5
Calculated p.d., millivolts ..	15.0	16.0	14.5	13.0	8.5	7.0	4.5	4.0	3.0	1.5
Osmotic pressure, mm. ...	9	16	29	33	25	22	19	18	16	14

*Effect of pH on p.d. and Osmotic Pressure of 1 Per Cent Globulin Acetate in 0.01 M Sodium Acetate.*

pH inside.....	4.63	4.34	4.02	3.69	3.35					
pH outside.....	4.61	4.31	4.01	3.63	3.31					
Observed p.d., millivolts ..	1.0	1.5	2.0	2.0	3.0					
Calculated p.d., millivolts ..	1.0	2.0	1.0	4.0	2.0					
Osmotic pressure, mm. ...	6	6	7	9	11					

represent the differences between the E.M.F. readings obtained for the inside and outside solutions with the hydrogen electrode, corrected to 25°; in other words, the calculated p.d. values are calculated from the measurements of the hydrogen ion concentration according to the formula deduced by Donnan (which is identical with Nernst's formula).

The results of experiments on the effect of acidity on the p.d. are given in Table II.



The results show that with protein and acid alone the agreement between the observed and calculated values for the p.D. is fairly good except in the region near the isoelectric point, and the last experiment in Table II shows that agreement may be obtained in this range of pH also by the use of buffered solutions.<sup>9</sup>

The effect of salt concentration on the p.D. was tested by a few experiments with NaCl, CaCl<sub>2</sub>, and LaCl<sub>3</sub>, and the results are given in Table III. Again the agreement between the observed and calcu-

TABLE III.

*Effect of Different Chlorides on the p.D. and Osmotic Pressure of 1 Per Cent Globulin Chloride.*

Concentration of salt.	O	N/1,024			N/256			N/32		
Salt .....		NaCl	CaCl <sub>2</sub>	LaCl <sub>3</sub>	NaCl	CaCl <sub>2</sub>	LaCl <sub>3</sub>	NaCl	CaCl <sub>2</sub>	LaCl <sub>3</sub>
pH inside.....	3.68	3.58	3.60	3.55	3.82	4.10	3.80	3.66	3.69	3.57
pH outside.....	3.34	3.36	3.38	3.33	3.76	3.93	3.73	3.63	3.63	3.54
Observed p.D., millivolts ..	21.0	13.5	12.5	13.0	4.0	3.0	3.5	1.0	1.0	1.0
Calculated p.D., millivolts ..	20.5	13.0	13.0	12.5	4.0	10.0	4.0	1.5	3.5	1.5
Osmotic pressure, mm. ..	52	25	26	28	20	16	19	4	4	4

TABLE IV.

*Effect of pH on the p.D. and Osmotic Pressure of 1 Per Cent Sodium Globulinote.*

pH inside.....	9.20	9.98	10.47	11.05
pH outside.....	9.81	10.34	10.85	11.31
Observed p.D., millivolts.....	-32.0	-23.0	-18.0	-11.5
Calculated p.D., millivolts.....	-36.5	-21.0	-22.5	-16.0
Osmotic pressure, mm. ....	220	174	165	152

lated values for the p.D. is sufficient to show that the p.D. is due to the Donnan equilibrium. Moreover, the different chlorides, at equivalent chloride ion concentrations, have identical effects in depressing the p.D. This proves that here too, as in the case of other proteins, the p.D. is affected only by the ion of opposite charge to that of the protein ion.

Table IV gives results which indicate that the predictions of the Donnan theory are fulfilled on the alkaline side of the isoelectric

<sup>9</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 617.

point. Here the pH inside was found to be less than the pH outside, and the observed P.D. was opposite in sign to that observed with the acid solutions, the inside solution now being negative with respect to the outside. While the quantitative agreement between the observed and calculated values is not so good as on the acid side, this is probably due to the effect of CO<sub>2</sub> from the air on the pH values. The solutions were protected by soda lime tubes while osmotic equilibrium was being reached, but were open to the air during the P.D. measurements.

#### IV.

##### *Osmotic Pressure.*

The osmotic pressure of these solutions was affected in the same sense by changes in pH as in the case of other proteins. On the acid side the osmotic pressure increased from small values near the isoelectric point to a maximum in the neighborhood of pH 3, and decreased with further increases in acidity. This is qualitatively in accord with the Donnan theory. The actual calculation of the osmotic pressure from the hydrogen ion measurements, however, gave values which were much higher than those observed, the maximum in the case of globulin chloride being over 3.5 times as high as that observed. The explanation for this discrepancy may lie in the existence of large aggregates in the globulin solutions. Loeb has shown<sup>10</sup> that the presence of undissolved particles of gelatin in place of dissolved gelatin has very little effect on the P.D. of a gelatin chloride solution, but that it materially decreases the osmotic pressure; the undissolved particles appearing to have but a slight share in the osmotic pressure as measured by the manometer. Since many of the globulin solutions were very opaque it is quite probable that much of the globulin was in the form of particles which produced no measurable osmotic pressure. However, a second preparation of globulin gave nearly clear solutions in HCl which still had an osmotic pressure much less than that calculated from the pH measurements.

The small osmotic pressure which was measured, however, was affected by salts in the way predicted by the theory. Table III shows that equivalent concentrations of different chlorides had the

<sup>10</sup> Loeb, J., *J. Gen. Physiol.*, 1921-22, iv, 769.

same effect in decreasing this osmotic pressure, the decrease being evidently dependent on the concentration of the ion of opposite sign of charge to that of the protein ion.

The osmotic pressure observed in the experiments with alkali was more nearly of the same magnitude as that calculated on the basis of the Donnan theory, indicating that the sodium globulinate probably contained fewer large aggregates than the globulin chloride. Quantitatively, however, the agreement was poor; possibly this may be due to the presence of sodium carbonate.

#### SUMMARY.

1. The globulin prepared from ox serum by dilution and precipitation with carbon dioxide has been found, by electrometric titration experiments, to behave like an amphoteric electrolyte, reacting stoichiometrically with acids and bases.

2. The potential difference developed between a solution of globulin chloride, phosphate, or acetate and a solution of the corresponding acid, free from protein, separated from the globulin by a collodion membrane, was found to be influenced by hydrogen ion concentration and salt concentration in the way predicted by Donnan's theory of membrane equilibrium. In experiments with sodium globulinate and sodium hydroxide it was found that the potential difference could be similarly explained.

3. The osmotic pressure of such solutions could be qualitatively accounted for by the Donnan theory, but exhibited a discrepancy which is explicable by analogy with certain experiments of Loeb on gelatin.

4. The application of Loeb's theory of colloidal behavior, which had previously been found to hold in the case of gelatin, casein, egg albumin, and edestin, has thus been extended to another protein, serum globulin.

The writer's thanks are due to Dr. Jacques Loeb for his suggestion and guidance of this work.

# THE EXCRETION OF CARBON DIOXIDE BY RELAXED AND CONTRACTED SEA ANEMONES.

By G. H. PARKER.

(*From the Zoological Laboratory of Harvard University.*)

(Received for publication, April 15, 1922.)

## I.

### INTRODUCTION.

It is well known that clams, oysters, and other invertebrates, provided with smooth muscle may contract and remain so even against considerable resistance for days or even weeks at a time. Oysters are said to keep their valves closed against the action of the ligament for from 20 to 30 days. Sea anemones are in this respect almost equally remarkable, for they have been known to remain contracted for days or even weeks.

As early as 1878 Coutance pointed out that the adductor muscles of the pelecypods were composed of two parts, one of smooth fibers, and the other of cross-striated. The quick closing of the valves was accomplished by the cross-striated muscle, and the continued closure was maintained by the smooth muscle. Marceau (1909) compared the action of these muscles to that of a cog-wheel with a catch. In such a mechanism energy was required to move the wheel, but by means of the catch the wheel could be held in any position without further work. Essentially, the same view was advanced by von Uexküll (1909) who found that among other invertebrate structures the spines of sea urchins were provided with two classes of muscles, one for motion, and the other for holding. Muscles which have this peculiar capacity of holding have been called tonus muscles and Bethe (1903, 1911), as a result of his study of snails and of clams, declared that in tonus muscle energy is consumed in passing from one state to another but not in maintaining a given state. The body muscles of the large marine snail *Aplysia* remained contracted 10 days in the

animal without food, a condition inconceivable on the assumption that contraction implies a characteristically high rate of metabolism. A similar conclusion was drawn by Bethe from the fact that fresh water mussels remained contracted under load for as long as 25 days and yet the substance of these animals was no more diminished than that of those that were normally resting. Parnas (1910), who studied the absorption of oxygen and the excretion of carbon dioxide in clams and other pelecypods, found that at maximum contraction and under high load the adductor muscles of these animals showed no increase of metabolism. Hence, the experimental evidence, so far as it goes, favors the view that in the so called tonus muscles the energy-consuming process is merely that of changing from one state of shortening or elongation to another, and that the maintenance of a state once reached is not at the expense of energy, but is dependent upon some such mechanical device as would be represented by a hook or a catch in such a mechanism as a cog-wheel (Bayliss, 1915).

The muscles of sea anemones, so far as contraction is concerned, exhibit all the properties of the so called tonus muscles. The common sea anemone of the New England coast, *Metridium marginatum* Edw., may be said to average about 6 to 8 cm. in height and 3 to 4 cm. in diameter. Such an animal in a fully expanded condition may have a volume of about 85 cc. On passing into full contraction it will discharge sea water to the extent of about 70 cc. and the residual 15 cc., representing for the most part the living substance of the animal, may remain in a contracted, compact form, for days at a time. The muscles concerned with this contraction, which are all of a very primitive, smooth variety, may shorten to as much as one-tenth their greatest length and remain persistently in this shortened state. Sea anemones afford, therefore, very convenient material for testing the question of the relative activity of tonus muscles at various stages of contraction.

As previous workers have shown, the most convenient line of attack on this problem is the metabolic one, and of the evidences of metabolism the excretion of carbon dioxide is perhaps the easiest one to determine. The question resolves itself as follows: In a given sea anemone what is the output of carbon dioxide during the four states of relaxation, of contraction, of passing into the relaxed state, and

of passing into the contracted state? Assuming that the amount of carbon dioxide excreted is a measure of the metabolism of the sea anemone, these four determinations ought to throw light on the nature of the activity of tonus muscle, for the contraction and relaxation of a sea anemone is the contraction and relaxation of its smooth muscles.

In making these determinations an Osterhout respiratory apparatus was employed. This apparatus has already been described and figured (Osterhout, 1918), and the method by which it was calibrated for this particular piece of work has already been published (Parker, 1922). Suffice it to say that the apparatus consists of a system of chambers and tubes whereby a closed circulation of air is maintained by means of a rubber pump provided with valves. From a chamber in which the organism is contained, and consequently in which the carbon dioxide is produced, the air is carried either directly to a tube filled with an indicator solution, or indirectly to it, in that on the way to the indicator the air is passed through a U-tube filled with fragments of sodium hydroxide for the absorption of carbon dioxide. In the first instance, the air, carrying a definite proportion of carbon dioxide, bubbles through the indicator and gradually charges it with carbon dioxide, thus changing its tint; in the second, the air, freed from carbon dioxide, bubbles through the indicator and washes out the contained carbon dioxide, thus returning the indicator to its original tint. Whether the air will go directly or indirectly from the chamber in which the organism is kept to the indicator, is arranged by a system of pinch-cocks. From the indicator the air is returned through the rubber pump to the chamber in which the organism is kept. In taking readings with this apparatus, a record is made of the time in seconds necessary to change the color of the indicator by means of the carbon dioxide given out by the organisms from one standard tint to another. The indicator used in these tests was an aqueous solution of phenolsulfonephthalein, and the two standard tints were those represented by pH 7.36 and pH 7.78 (see Osterhout and Haas, 1918). In taking a reading, the apparatus was run with an appropriate current of air till the indicator corresponded in tint to that of the standard tube pH 7.78; the air direct from the organism was then passed through the indicator and the time necessary to change the

indicator from the tint characteristic for pH 7.78 to that for pH 7.36 was recorded. This rate of change was assumed to correspond to the rate at which the sea anemone gave out carbon dioxide, and was the desired figure. As these readings could be made every few minutes, for it takes only a short time to set and reset the apparatus, an almost continuous record of the carbon dioxide output of the sea anemone could be kept, a great advantage which the Osterhout apparatus has over those of other types of construction. As none of the previous workers on this problem had used methods presenting this advantage, it was hoped that novel results would be obtained thereby.

The first trials were made on sea anemones in expanded or contracted states in sea water, but it was soon evident that the carbon dioxide liberated by the sea anemone was not freely given up to the air by the sea water which, without doubt, acted as a buffer solution. Fortunately, however, *Metridium* is a sea anemone that naturally spends much time between tides and consequently out of water. Therefore, it could be suspended in the air without harm, and when tested in this position, its output of carbon dioxide was found to be continuous and relatively uniform.

The work was done at the Marine Biological Laboratory at Woods Hole, Mass., and I am under obligations to Professor F. R. Lillie, director of the laboratory, and his staff for many privileges and courtesies. I am also under obligations to Professor W. J. V. Osterhout for much help and advice in the use of his respiratory apparatus.

## II.

### OBSERVATIONS.

*A. Relaxed Sea Anemones.*—In measuring the amount of carbon dioxide excreted by relaxed specimens of *Metridium*, it was found best to hang the animal in the respiratory chamber rather than to allow it to lie on the floor of the chamber. By puncturing the foot of the sea anemone a cord could be passed inward through its mouth and out through its foot in such a way as to make a convenient loop by which the animal could be suspended from a hook in the rubber stopper of the respiratory chamber. Such a sea anemone hung like a mass of tough jelly in contact on all sides with the air of the

respiratory chamber. Occasionally the weight of the animal caused the cord to cut through the tissues and the whole animal fell to the bottom of the chamber, but in most instances the tissues were tough enough to resist the cord and the animal remained suspended during the test. The tissues of the sea anemone were too delicate to admit of much additional weighting so that the animals carried only their

TABLE I.

Times in seconds necessary to change the indicator solution in an Osterhout apparatus from pH 7.78 to pH 7.36 by the carbon dioxide excreted by relaxed specimens of *Metridium*, Nos. 1 and 2 separately, and 1 and 2 together. At the bottom of the table are given the averages of the times, the carbon dioxide excreted in hundred-thousandths of a milligram per second, the weight of the sea anemones in grams, and the weight of carbon dioxide in hundred-thousandths of a milligram secreted per second per gram weight of sea anemone.

No. of individual.....	1	2	1 and 2
Respiratory time, sec.....	484 372 466 382 420	461 438 392 401 410	207 209 231 190 228
Average time.....	424.8	420.4	213
CO <sub>2</sub> per sec., in 1/100,000 mg.....	3.0+	3.1-	6.0+
Weight, gm.....	0.5	0.5	1.0
CO <sub>2</sub> per sec., per gm.....	6.0+	6.1+	6.0+

own weight, and the muscles in contracting lifted that weight and no more. Sea anemones that had been thus hung soon discharged most of the sea water contained in their bodies, and tests were not made till this superfluous fluid had all been drained off. The sea anemone then hung as an elongated jelly-like mass surrounded on all sides by the air of the respiratory chamber, and in this relaxed condition observations were made on the rate at which it excreted carbon dioxide.

In some preliminary trials two relaxed sea anemones of the same weight, 0.5 gm., were tested separately and together with results



as shown in Table I. The average time necessary for the appropriate change in the indicator when the two animals were tested separately was approximately the same, 424.8 seconds for No. 1 and 420.4 seconds for No. 2. Each sea anemone must, therefore, have produced carbon dioxide at about the same rate. What this rate was in absolute terms can be ascertained by using the formula obtained from the calibration of the apparatus as already referred to (Parker, 1922). This formula is as follows:

$$\left(\frac{K}{T} = W\right)$$

in which  $K$  is the constant of the apparatus, in this particular instance 1,283.5,  $T$  is the time in seconds for the indicator change, and  $W$  is the weight of carbon dioxide delivered in hundred-thousandths of a milligram per second.

In individual No. 1, this rate proved to be 3.0+ and in No. 2, 3.1—hundred-thousandths of a milligram of carbon dioxide per second. When the two animals were tested together, the average time necessary to change the indicator was found to be 213 seconds which corresponds to the production of 6.0+ hundred-thousandths of a milligram of carbon dioxide per second. Thus, the two sea anemones together made the requisite change in the indicator in almost exactly half the time taken by either alone, and gave evidence of excreting carbon dioxide at double the rate of the separate individuals. These tests not only yield results of importance in the respiration of *Metridium*, but they also indicate the relative accuracy of the apparatus.

Other relaxed sea anemones were tested for their production of carbon dioxide with the results shown in Table II. Here it will be seen that the heavier the specimen, the shorter the respiratory time, and consequently the larger the calculated amount of carbon dioxide excreted in unit time. At the bottom of the table the amount of carbon dioxide in hundred-thousandths of a milligram excreted per second by a gram weight of sea anemone is given. This amount varies from 4.8+ to 5.9+ or, if all the results in this paper are included, from 4.4 to 6.1. The average of all these measurements is 5.43—and it may, therefore, be concluded that a relaxed *Metridium* ex-

cretes for each gram of living substance about 5.5 hundred-thousandths of a milligram of carbon dioxide per second, a figure very close to that for the clam, *Venus*, as calculated from the observations of Parnas (1910), namely, 2.3 hundred-thousandths of a milligram of carbon dioxide per second per gram of living substance.

TABLE II.

Times in seconds necessary to change the indicator solution in an Osterhout apparatus from pH 7.78 to pH 7.36 by the carbon dioxide excreted by relaxed specimen of *Metridium*. At the bottom of the table are given the averages of the times, the carbon dioxide excreted in hundred-thousandths of a milligram per second, the weight of the sea anemones in grams, and the weight of carbon dioxide in hundred-thousandths of a milligram excreted per second per gram weight of sea anemone.

No. of individual.....	3	4	5	6	7
Respiratory time, sec.....	204 171 214 200 214	205 200 202 207 198	89 103 96 92 100	71 58 59 63 66	38 42 38 43 40
Average time.....	200.6	202.4	96.0	63.4	40.2
CO <sub>2</sub> per sec., in 1/100,000 mg.....	6.4—	6.3+	13.4—	20.2+	31.9+
Weight, gm.....	1.2	1.3	2.7	4.2	5.4
CO <sub>2</sub> per sec., per gm.....	5.3+	4.8+	5.0—	4.8+	5.9+

*B. Contracted Sea Anemones.*—When a sea anemone is suspended in an Osterhout apparatus, it quickly assumes the relaxed condition and the records given in the preceding section were taken from animals in this state. The contracted condition can be easily induced by moving the glass vessel in which the sea anemone is suspended back and forth a little, whereby the sea anemone is made to swing and strike on the sides of the vessel. This movement of the vessel can be accomplished without disturbing the rest of the apparatus in consequence of the rubber connections between the vessel and the other parts. By thus stimulating the sea anemone from time to time, it can be kept in a condition of continuous and extreme contraction.

# Urinary Excretion Kinetics for Evaluation of Drug Absorption II\*

## Constant, Rate-Limited Release of Tetracycline After Ingestion By Humans

By EINO NELSON

*In vivo* absorption studies in humans of a composition of tetracycline and mucic acid, it was found that absorption was rate-limited by the *in vivo* dissolution rate of this composition when it was administered as thin pellets whose surface area could be assumed to have remained constant after ingestion. The rate-limiting *in vivo* dissolution rate was calculated to be about 2 mg. tetracycline as the hydrochloride per sq. cm. of composition per hour.

THIS PAPER presents and discusses the results of studies on tetracycline in which constant, rate-limiting release of drug was established in absorption from the gastrointestinal tract of humans. The studies were conducted with a composition of tetracycline and mucic acid (TMAC). The bases for interpretations on the excretion rate measurement used in evaluation were described in the first paper of this series (1).

### EXPERIMENTAL

**Conduction of Tests, Assay, and Preparations of Dosage Forms.**—All aspects of these procedures were essentially the same as previously described (2). Drug was ingested by humans in apparent good health on fasting stomachs. In one series of tests, each dose of drug consisted of two thin pellets or disks about 0.95 cm. in diameter and 0.15 cm. thick contained in 000 hard gelatin capsules with sodium bicarbonate added to insure separation of the pellets in the stomach after ingestion. The dose of tetracycline was  $200 \pm 10$  mg. in each test in terms of the equivalent of the hydrochloride salt. The compaction process used in preparation of the pellets insured their solution from exposed faces after ingestion rather than disintegration. The geometry of the pellets indicated that release of drug should be at a constant rate in dissolution medium of constant composition, since the surface area of their faces was much larger than their peripheral surface area. In another test series using the same subjects, TMAC equivalent to 125 mg. of the activity of tetracycline hydrochloride was taken in a raspberry-flavored aqueous suspension.

Collection times for urine specimens and pertinent data on subjects are indicated in the tabulated data that follow later in this report.

**Tetracycline Mucic Acid Composition.**—This material was prepared by mixing stoichiometric quantities of tetracycline hydrochloride and disodium mucate in aqueous solution, collecting and washing the resulting precipitate, and drying under vacuum at room temperature over sulfuric acid. Repeated microbiological assays on several batches of composi-

tion indicated that this material possessed an average of 87% of the activity of the tetracycline hydrochloride used in its preparation. The theoretical potency of a composition of two moles tetracycline to one mole of mucic acid is 88%. The composition possessed a water solubility at 25° of about 0.6 mg./ml. by microbiological assay and saturated solutions of the composition at 25° assumed a pH of 3.20 as measured on a Beckman model G pH meter, freshly standardized at pH 4.01. The nature of the interaction between tetracycline and mucic acid is the subject of continuing investigation (3).

### RESULTS AND DISCUSSION

**Excretion Data.**—The cumulative amounts of tetracycline excreted to various times after ingestion of 200 mg. (HCl equivalent) of TMAC in the constant surface pellets is shown in Table I. The mean cumulative amounts of tetracycline excreted to various times after ingestion of 125 mg. (HCl equivalent) of the TMAC in suspension were 2.5, 8.0, 15.2, 20.8, 27.1, and 32.9 mg. tetracycline hydrochloride equivalent at one, two, three, four, six, and eight hours, respectively. The individual data will be reported in another paper (3).

**Interpretation of Excretion Data.**—When cumulative plots of amounts of tetracycline excreted were constructed from the data collected on individual test subjects, it was found that in two cases the slope of the plots assumed a constant value between two and three hours after drug ingestion. This is shown in Fig. 1 for subjects B and T. When the averaged data from the six subjects participating in this particular test were plotted, the same occurrence was noted as shown in the lower curve of Fig. 2. These results indicated that an equilibrium was established between tetracycline absorption, distribution, and excretion in accordance with considerations previously discussed concerning the kinetic principles involved (4). As previously mentioned, due to the design of the pellet dosage form, the surface area exposed to medium at absorption sites could be expected to remain relatively constant during dissolution leading to constant rate release and hence establishment of the equilibrium condition noted. The slopes of excretion curves were 3.8 and 3.4 mg./hr., respectively, for subjects B and T and 4.5 mg./hr. for all six subjects combined. These values, divided by the fraction of tetracycline in circulation that was

\* Received July 6, 1959, from the School of Pharmacy, University of California, San Francisco 22.

Supported in part by a grant-in-aid from The Squibb Institute For Therapeutic Research.

TABLE I.—CUMULATIVE MG. TETRACYCLINE AND ML. URINE EXCRETED TO VARIOUS TIMES FROM TMAC IN PELLET FORM<sup>a</sup>

Subject <sup>b</sup>	mg. Excreted, Hr								ml. Urine, Hr.							
	1	2	3	4	6	8			1	2	3	4	6	8		
E (39-77)	2.7	9.2	16.9	23.0	33.5	42.8			110	340	500	590	780	970		
S (33-57)	0.8	5.0	12.0	20.1	31.6	38.9			90	260	420	590	710	870		
B (28-82)	0.4	2.7	5.8	9.3	16.6	23.9			40	80	120	160	250	350		
T (23-75)	0.3	2.1	5.0	8.3	15.0	20.5			60	120	180	280	360	430		
N (21-77)	0	0.4	1.1	2.2	4.8	8.0			310	350	380	410	480	550		
H (26-73)	0.2	0.9	5.3	12.3	29.4	49.4			50	160	350	580	720	860		
Mean	0.7	3.4	7.7	12.5	21.8	30.6			110	218	325	435	550	672		

<sup>a</sup> Dose equivalent to 200 ± 10 mg. tetracycline hydrochloride. Excreted amounts are also in terms of hydrochloride equivalents.

<sup>b</sup> Quantities in parentheses are subjects' age in years followed by weight in Kg.

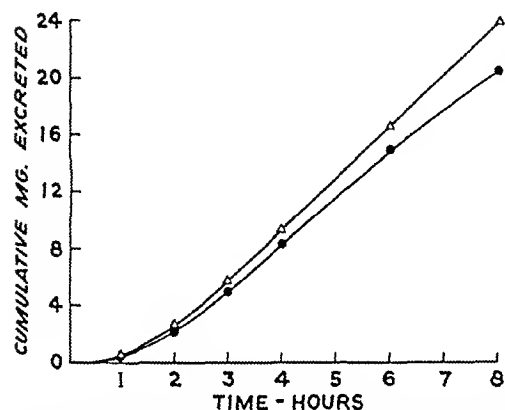


Fig. 1.—Constant excretion rate after ingestion of tetracycline mucic acid composition in constant surface pellets by subjects B and T. Solid circles, subject T; open triangles, subject B.

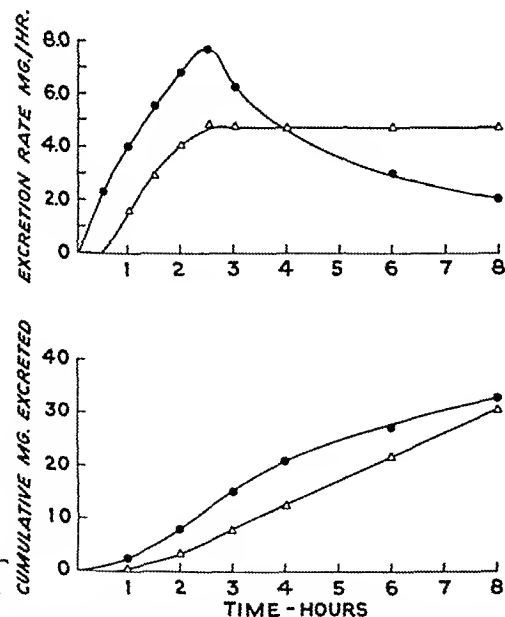


Fig. 2.—Lower pair of curves shows difference in cumulative amounts excreted when tetracycline mucic acid composition was given as a 125-mg. dose (HCl equivalent) in suspension, solid circles, and as constant surface pellets of this material in a 200-mg. dose (HCl equivalent), open triangles. Upper curves show excretion rates in the same cases.

excreted unchanged, gave the *in vivo* absorption rate (2).<sup>1</sup> In consideration of the conditions necessary for establishment of the equilibrium, these same rates must be equal to the *in vivo* dissolution rate of the pellets. Since the surface area of the pellet dosage was of the order of 3 sq. cm., the average *in vivo* solution rate was about 2 mg. tetracycline HCl/sq. cm./hr.

The constant rate release discussed persisted on the average for at least six hours after establishment, indicating that tetracycline, as TMAC, was available for dissolution and absorption in the gastrointestinal tract for at least the same period.

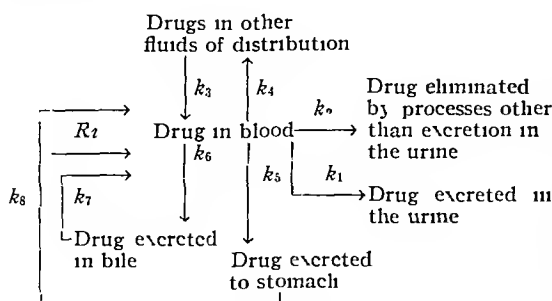
The upper curves of Fig. 2 which are plots of excretion rates indicate the nature of blood level curve that could be expected from the constant surface pellets of the TMAC under the conditions of this test. The corresponding cumulative amounts excreted and excretion rate *vs.* time curves for the 125-mg. dose (HCl equivalent) of the composition in suspension are included on both the lower and upper diagrams of Fig. 2 for comparative purposes. The higher excretion rates from the suspension preparation with a smaller dose of drug clearly indicate that *in vivo* solution rate was rate-limiting in absorption when the pellets were used and the correctness of the interpretation placed on the results.

The interpretation in this section concerning the estimated magnitude of *in vivo* dissolution and absorption rates from constant surface pellets are independent of the amounts of tetracycline that may have been involved in an excretion-absorption cycle via the bile. Tetracycline is found in the bile in high concentrations (5), but the volume of bile flowing is a small fraction of the volume of the other fluids of distribution and only small amounts would be expected to be involved in this cycle. The possibility also existed that tetracycline was involved in another excretion-absorption cycle via the stomach as a consequence of the pH-partition hypothesis (6). However, since an equilibrium between tetracycline absorption and excretion was apparently established, drug in these cycles was at equilibrium with drug in other fluids of distribution.

A generalized mathematical treatment to consider constant rate excretion from sustained release products presented by Campbell and co-workers (4) may be extended to include the bile and stomach cycles to show that this conclusion is correct. The absorption, distribution, and elimination of tetracycline may be portrayed by the following scheme when

<sup>1</sup> If this fraction was about 0.75, then multiplication of the excretion rates by 1.33 will give the absorption rates.

drug is released at a constant rate,  $R_i$ , at the absorption site



In the scheme shown the  $k$ 's with number subscripts are first order rate constants for the indicated transfer processes and may be considered to incorporate rate partition factors when necessary, e g., between drug in blood and drug in bile. The differential equations that describe distribution in the above scheme are

$$dA_b/dt = R_i + k_3A_d - k_4A_b - k_2A_b - k_1A_b - k_5A_b - k_6A_b + k_7A_i + k_8A_e \quad (\text{Eq } 1)$$

$$dA_d/dt = k_4A_b - k_3A_d \quad (\text{Eq } 2)$$

$$dA_e/dt = k_5A_b - k_1A_e \quad (\text{Eq } 3)$$

$$dA_i/dt = k_6A_b - k_7A_i \quad (\text{Eq } 4)$$

$$dA_e/dt = k_1A_b \quad (\text{Eq } 5)$$

In the above set of equations  $A_b$ ,  $A_d$ ,  $A_i$ ,  $A_e$ , and  $A_e$  are, respectively, the amounts of drug in blood, other fluids of distribution, bile, stomach, and amount excreted. Since constant rate excretion was observed in the experiments, the amount of drug in blood  $A_b$ , must have remained at a constant value as a consequence of Eq 5. With  $A_b$  constant,  $dA_b/dt = 0$ . Further,  $dA_d/dt = 0$ ,  $dA_e/dt = 0$ , and  $dA_i/dt = 0$ . Since conditions other than this one would not allow  $dA_b/dt = 0$ , then

$$k_4A_b = k_3A_d \quad (\text{Eq } 6)$$

$$k_5A_b = k_1A_e \quad (\text{Eq } 7)$$

$$k_6A_b = k_7A_i \quad (\text{Eq } 8)$$

and

$$0 = R_i - (k_1 + k_2)A_b$$

or

$$A_b = R_i/(k_1 + k_2) \quad (\text{Eq } 9)$$

Equation 5 may then be written as

$$dA_e/dt = (k_1/k_1 + k_2)R_i \quad (\text{Eq } 10)$$

The coefficient of  $R_i$  on the right hand side of Eq 10 has previously been noted to be equal to  $f$ , the fraction of drug excreted unchanged (4) so

$$\text{Constant excretion rate} = f R_i \quad (\text{Eq } 11)$$

More complex distribution schemes than the above could be written. With constant excretion rate, Eq 11 would still be obtainable from the equations describing the more complex scheme. For example,

'drug in blood could be considered to exchange between several more compartments without affecting the generality and conclusions obtained from the scheme discussed in detail here.

**Absorbability of TMAC in In Vivo Solution Rate-Limited Trials**—The water solubility of TMAC was only about twice that possessed by tetracycline. In solution rate limited absorption trials with the latter material with the same subjects and under the same conditions as used for TMAC trials, the mean maximum excretion rate observed was of the order of 1.8 mg/hr followed by a steadily decreasing rate from about two hours after ingestion to the end of the tests (2). This contrasts to the constant rate of about 4.5 mg/hr persisting for at least six hours after its establishment, two hours after ingestion of the same dose of TMAC. Since in both cases absorption was rate limited by *in vivo* solution rate, the TMAC was capable of nearly constant rate dissolution after ingestion and the site of this dissolution was probably in the small intestine. The ability of solid TMAC to dissolve *in vivo* at a fair rate even though its water solubility was of a low order, was no doubt due to a physical chemical property possessed by it but not by tetracycline.

Tetracycline possesses two acidic functions and one basic function (7). At hydrogen ion concentrations likely to be found in intestinal fluids, tetracycline would exist either as the zwitter ion, since the pH of its isoelectric point is about 5.5, or as the anion to a lesser degree, depending on the pH of the fluids. This means that the diffusion layer surrounding undissolved tetracycline would possess a pH near that of intestinal medium. No strong driving force for dissolution would exist under these conditions since the concentration in the diffusion layer would be a small value. On the other hand, the tetracycline mucic acid composition could be expected to possess an intrinsically strong driving force for dissolution in the intestinal fluids. The distinct acidic reaction possessed by the saturated solution of the composition as mentioned earlier in this report would necessitate a neutralization reaction to take place at the diffusion layer<sup>3</sup> of undissolved material. Since the hydrogen ion concentrations of intestinal and duodenal fluids are maintained by a bicarbonate buffer system, carbon dioxide would be evolved in this reaction. The gas evolution should hasten solution by facilitation of exchange of material between the diffusion layer and medium. This effect was observed *in vitro* by immersing one of the pellets used in the *in vivo* trials in a simulated intestinal fluid containing a small amount of sodium bicarbonate.

## REFERENCES

- (1) Nelson E and Schaldemose I. THIS JOURNAL 48, 489(1959).
- (2) Nelson E. *ibid* 48, 96(1959).
- (3) Nelson E. U.S. pat. pending.
- (4) Campbell J A, Nelson E and Chapman D G. *Can. Med. Assoc. J.* 81, 15(1959).
- (5) Pulaski E J and Isokane R K. *Antibiotic Med. & Clin. Therapy* 4, 408(1957).
- (6) Shore P A, Brodie B B and Hogben C A M. *J. Pharmacol. Exptl. Therap.* 119, 361(1957).
- (7) Conover L H. Symposium on Antibiotics and Mold Metabolites. The Chemical Society, London, England, 1956, p. 48.

<sup>2</sup> Based on the strongest acidic function.

<sup>3</sup> The thin layer of fluid that always surrounds a dissolving solid.

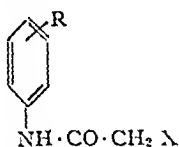
# Studies on Local Anesthetics XXII\*

## The Basic Aroxy- and Aralkoxyacetanilides

By ALOIS BOROVANSKÝ, ALEŠ SEKERA†, and ČENĚK VRBA‡

The synthesis of nine isomeric phenoxy, benzyloxy, and phenylethoxy substituted diethylaminoacetanilides is reported. The activity in both surface and infiltration anesthesia and acute toxicity tests was determined.

CONTINUING OUR STUDY of the substituted acetanilides with anesthetic properties, we have prepared a new series of phenoxy, benzyloxy, and phenylethoxy derivatives of general formula I. Their structures are evident from Table II



I, X =  $-\text{N}(\text{C}_2\text{H}_5)_2$

II, X =  $-\text{Cl}$

R =  $\text{C}_6\text{H}_5\text{O}$ ,  $\text{C}_6\text{H}_5\text{CH}_2\text{O}$ ,  $\text{C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{O}$

We have been led to this mode of substitution by observation of the interesting pharmacological properties of the diethylaminoethyl esters of aroxy- and aralkoxycarbanilic acids, the properties of which we have already reported (1)

The compounds were prepared according to the method previously described (2) the chloroacetanilides (II), obtained from the respective aryl amines, are converted to the corresponding basic aroxy- and aralkoxyacetanilides (I) by treatment with diethylamine

### EXPERIMENTAL<sup>1</sup>

**Aromatic Amines.**—These have been prepared by catalytic hydrogenation of the corresponding nitro compounds over Raney nickel according to the method described in a previous paper (1) The physical properties and the yields of the substances prepared correspond to the values there indicated

**Chloroacetanilides (II).**—These have been prepared by the reaction of chloroacetyl chloride and the corresponding aryl amines in acetone in the presence of sodium carbonate (2) For the final reaction the crude products were used The samples for the analysis were purified by crystallization from petroleum ether. The yields, the physical properties,

and the analyses of the substances prepared are indicated in Table I.

**Diethylaminoacetanilides (I; S250 - S258)**—These have been prepared according to the method previously used (2) by the reaction of the corresponding chloroacetanilides with the diethylamine in anhydrous benzene The hydrochlorides were precipitated from anhydrous ether solutions of bases by addition of ethereal hydrogen chloride. The yields, the physical properties, and the analyses of the substances prepared are included in Table II

TABLE I.—SUBSTITUTED CHLOROACETANILIDES

No	R	Yield, %	M <sup>c</sup> p, °C	N, % Calcd	N, % Found
1	$2\text{-C}_6\text{H}_5\text{O}$	97	77	5.35	5.43
2	$3\text{-C}_6\text{H}_5\text{O}$	67	107 <sup>a</sup>	5.35	5.35
3	$4\text{-C}_6\text{H}_5\text{O}$	99	101	5.35	5.42
4	$2\text{-C}_6\text{H}_5\text{CH}_2\text{O}$	95	103	5.08	4.95
5	$3\text{-C}_6\text{H}_5\text{CH}_2\text{O}$	94	120	5.08	5.14
6	$4\text{-C}_6\text{H}_5\text{CH}_2\text{O}$	84	148 <sup>b</sup>	5.08	5.12
7	$2\text{-C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{O}$	96	59	4.83	4.60
8	$3\text{-C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{O}$	95	63	4.83	4.99
9	$4\text{-C}_6\text{H}_5\text{CH}_2\text{CH}_2\text{O}$	76	124 <sup>c</sup>	4.83	5.02

<sup>a</sup> From dilute ethanol

<sup>b</sup> Purification of the sample for analysis 100 cc of the anhydrous 5% chloroform solution was filtered through a column of 10 Gm of aluminum oxide (activity between I and II) and was then eluted by 300 cc of the same solvent. The product obtained by evaporation of the eluate was crystallized from dilute ethanol

<sup>c</sup> From ethanol

### PHARMACOLOGY<sup>2</sup>

The relative activity of the compounds in surface anesthesia (rabbit cornea, 0.01 M cocaine as standard) and infiltration anesthesia (intradermal application to guinea pigs, 0.02 M procaine as standard) was calculated from the molar concentration, experimentally found to give the same effect as the standard. The method has been described in detail by Vrba and Sekera (3, 4)

The toxicity was studied according to the method of Karber (5) by determining the LD<sub>50</sub> in white mice (strain H) by subcutaneous injection.

The results are presented in Table III.

### DISCUSSION AND SUMMARY

Nine isomeric diethylaminoacetanilides, substituted in the benzene nucleus by a phenoxy, benzyloxy, and phenylethoxy group were prepared.

<sup>2</sup> We are obliged to Miss M. Soukupová and A. Nováková Niplova for technical assistance with the pharmacological tests

\* Received April 16, 1959, from the Department of Pharmaceutical Chemistry, Masaryk University Brno, Czechoslovakia

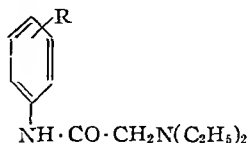
† Paper XXI of this series. THIS JOURNAL 48, 402 (1959)

‡ Inquiries regarding this article should be sent to A. Sekera Present address: Laboratoire de Pharmacologie Service de Chimie 21 rue de l'Ecole de Médecine Paris VI France

§ Department of Pharmacology School of Veterinary Medicine Brno, Czechoslovakia

¶ All melting points were determined on a Kofler block and are corrected. Microanalyses were carried out by Mrs. Klenova Parolková

TABLE II—SUBSTITUTED DIETHYLAMINOACETANILIDES



No	R	Base		Hydrochloride			
		Yield, %	M <sub>p</sub> , °C	M <sub>p</sub> , °C	N, %	Cl, %	
S 250	2-C <sub>2</sub> H <sub>5</sub> O	72		128	Calcd 8.39	Found 8.36	10.59
S 251	3-C <sub>2</sub> H <sub>5</sub> O	65		131 <sup>5a</sup>	8.39	8.30	10.59
S 252	4-C <sub>2</sub> H <sub>5</sub> O	56		123	8.39	8.53	10.59
S 253	2-C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> O	61		125 <sup>b</sup>	8.01	8.10	10.17
S 254	3-C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> O	76		145	8.01	8.24	10.17
S 255	4-C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> O	78	63 <sup>c</sup>	123	8.01	7.85	10.17
S 256	2-C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> CH <sub>2</sub> O	79		147-149	7.72	7.97	9.77
S 257	3-C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> CH <sub>2</sub> O	82		150	7.72	7.55	9.77
S 258	4-C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> CH <sub>2</sub> O	81	59 <sup>d</sup>	114	7.72	7.76	9.77

<sup>a</sup> Before the precipitation of the hydrochloride, the benzene solution of the anhydrous base (10.50) was filtered through a column of 15 Gm. of aluminum oxide (activity between I and II) and eluted with 300 cc. of the same solvent. The hydrochloride was crystallized from benzene ether.

<sup>b</sup> From benzene-ether.

<sup>c</sup> Anal.—Calcd. for C<sub>19</sub>H<sub>21</sub>O<sub>2</sub>N<sub>2</sub> (312.4) N, 8.97. Found N, 8.93.

<sup>d</sup> Anal.—Calcd. for C<sub>20</sub>H<sub>23</sub>O<sub>2</sub>N<sub>2</sub> (326.4) N, 8.58. Found N, 8.67.

TABLE III—PHARMACOLOGY OF SUBSTITUTED DIETHYLAMINOACETANILIDES

Substance	Relative Activity		LD <sub>50</sub> mg./Kg.
	Surface Anesthesia	Infiltration Anesthesia	
S 250	0.34	1.6	3,150
S 251	7.4	6.3	1,800
S 252	2.2	3.2	690
S 253	0.5	1.3	4,800
S 254	3.7	4.7	940
S 255	5.3	7.8	1,100
S 256	1	0.5	(6,000)
S 257	0.8	2	7,200
S 258	11	6.9	1,550
S 202 (lidocaine)	0.24	1.4	365
Cocaine	1	3.6	125
Procaine	0.15	1	630

pared and tested pharmacologically. The local anesthetic activities in surface and infiltration anesthesia and the acute toxicity were determined. It was found that most of the compounds are more active than cocaine in surface anesthesia and procaine in infiltration anesthesia. It was also found that they are, at the same time, up to ten times less toxic than these two standards.

From the results obtained, the following conclusions concerning the relations between the structure and the activity may be drawn:

1. If the activity of the substances prepared is compared with that of the model substance lidocaine<sup>3</sup> (S 202), it is to be noticed that lipophil-

ization of the molecule by the substitution of the phenoxy, benzyloxy, and phenylethoxy group generally increases the activity. This increase is especially evident in surface anesthesia.

2. The substitution in the *ortho* position was seen in every case to be the least advantageous. Substitution of the phenoxy radical in the *meta* position produced the greatest increase in activity, whereas in the benzyloxy and phenylethoxy series the compounds substituted in the *para* position are most active.

3. The appreciably lower toxicity of these compounds in comparison with lidocaine (S 202) is surprising and significant. This reduction in toxicity affected by aroxylation and aralkoxylation as illustrated by the compounds described in this paper, is more pronounced than in a series of closely related basic carbamates reported previously (1). It is interesting to notice a certain parallel between the anesthetic activity and the toxicity; the least active substances are also usually the least toxic.

## REFERENCES

- (1) Sova, J., Sekera, A., and Vrba, Č., *Chem. listy*, **51**, 2339(1957), *Experientia*, **13**, 495(1957).
- (2) Borovanský, A., Sekera, A., and Vrba, Č., *This Journal*, **48**, 402(1959).
- (3) Vrba, Č., and Sekera, A., *Arch. intern. pharmacodynamie*, **118**, 155 (1959).
- (4) Roth, Z., *ibid.*, **118**, 289(1959).
- (5) Kärber, G., in Burn, J. H., "Biologische Auswertungs methoden," Springer-Verlag, Berlin, Germany, 1937, p. 27.

<sup>3</sup> The trade name for lidocaine is Xylocaine.

## Notes

# A Note on the Application of Radioactive Isotopes to Studies of Drug Deterioration\*

By ROBERT F. SCHIFFMAN† and JOHN E. CHRISTIAN

THE PROBLEM of determining the stability of a new drug or dosage form has long been a vexing one to the drug manufacturer. Great competition demands that the determination be carried out as quickly and as efficiently as possible. The general procedure followed at the present time is to place the drug under accelerated aging conditions and then to test for purity by ordinary chemical and analytical means. The results thus obtained are then applied to basic kinetic principles, in a manner similar to that outlined by Garrett and Carper (1). In this manner it is possible to predict shelf life.

The use of radioactive isotopes and tracer techniques suggests itself as a method of studying stability and shelf life since it offers great sensitivity and ease of detection, even in complex mixtures. In this work the application of this technique to the study of hydrolysis of C-14 carboxyl acetylsalicylic acid is shown.

### EXPERIMENTAL

**The Electrophoretic Separation of Salicylic Acid and Acetylsalicylic Acid.**—C-14 carboxyl salicylamide was converted to C-14 carboxyl salicylic acid by alkaline hydrolysis using sodium hydroxide. The C-14 carboxyl acetylsalicylic acid was prepared using the method of Richter (2). This latter compound showed a specific activity of 0.1  $\mu\text{c./mg.}$  All separations were carried out employing the Reeco apparatus.<sup>1</sup> The separations were performed on Schleicher-Schuell 2045A filter paper strips. A buffer mixture composed of citrate-HCl, pH 4.0, ionic strength 0.40, was employed (3). Either 0.01 ml. or 0.02 ml. of the compounds to be separated were applied to the negative side of the strip. The strips were then run in an open strip manner, i. e., the strips were suspended on three plastic bridges so that no part of the strips touched the table of the electrophoresis apparatus and the ends hung free in the buffer. The entire apparatus was then enclosed with the plastic cover furnished with the apparatus, and made airtight by sealing the openings with Scotch electrical tape No. 33.<sup>2</sup> Electrophoresis was run for ten hours at 200 volts which produced a current of approximately 5 ma.

The spots were detected on the strips by either spraying with diazotized *p*-nitroaniline or by scanning with either the Actigraph apparatus<sup>3</sup> or the

Forro apparatus.<sup>4</sup> The narrowest slit opening was used with each apparatus.

Positive identification of the curves of the radioactive materials was made by comparison to controls of unlabeled salicylic and acetylsalicylic acids which were run along with these strips. The spots on the control strips were located by spraying with diazotized *p*-nitroaniline.

It was found that acetylsalicylic acid and salicylic acid could be separated by means of electrophoresis. However, the method used here was found not to be quantitative, since large sample losses, as shown by a decrease in total radioactivity, were found.

The reason for the loss of sample in this procedure is unexplained, although a possible explanation is that the passage of current may cause some decarboxylation of the C-14 carboxyl salicylic acid.

Thus, the separations performed as a part of the kinetic study of aspirin described here are qualitative only.

**Study of the Hydrolysis of C-14 Carboxyl Acetylsalicylic Acid.**—One hundred milliliters of a solution of C-14 carboxyl acetylsalicylic acid were prepared in Sørensen citrate-sodium hydroxide buffer of pH 6.7 (4), such that it had a concentration of 6 mg./ml. One-half milliliter of solution was then placed in each of sixty 1-ml. rubber-capped vials.<sup>5</sup> Twenty vials were placed in each of three constant temperature baths, set at 25, 42 and 60°,  $\pm 0.1^\circ$ . The vials were removed from the baths, one at a time, at half-hour intervals. The last two vials in each bath were removed at intervals of approximately twenty-four hours. Upon removal the vials were immediately placed in ice water and stored in a refrigerator.

Twenty lambda of each sample was spotted on a strip and the strips were run, eight at a time, in the electrophoresis apparatus. The strips were then analyzed for radioactivity by scanning with the Forro radiochromatograph scanner. The graphs thus obtained were then examined for the breakdown of acetylsalicylic acid and the formation of the products of deterioration.

The results obtained after subjection of several of the C-14 carboxyl acetylsalicylic acid samples to 60° are shown in Fig. 1. These graphs represent both the distance of travel of the radioactive materials and the relative activity of the spots. The activities are related directly to the concentrations of the materials; thus it is possible to follow the course of breakdown of the acetylsalicylic acid and the subsequent formation of salicylic acid.

\* Received August 21, 1959, from the Research Laboratories of the Bionucleonics Department, Purdue University.

† Present Address: DCA Food Industries, Inc., 45 West 36th St., New York, N. Y.

Presented to the Scientific Section, A. P. H. A., Cincinnati Meeting, August 1959.

<sup>1</sup> Model No. E-800-2, Research Equipment Corp., Oakland, Calif.

<sup>2</sup> Minnesota Mining and Manufacturing Co., St. Paul, Minn.

<sup>3</sup> Actigraph apparatus, model No. C-100, Nuclear Instrument and Chemical Corp., Chicago, Ill., 1.4 mg/cm<sup>2</sup>, mica window.

<sup>4</sup> Forro radiochromatograph scanner, model ACSHL, the Forro Scientific Co., Evanston, Ill., windowless, gas-flow counter.

<sup>5</sup> Wheaton injectable vials, No. 150-1, star line, with No. 1 sleeve stoppers.



*Methods of Analytical Histology and Histo-Chemistry*

By EDWARD GURR. The Williams and Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1959. xv + 327 pp. 15 x 24 cm. Price \$13.

This book describes in concise detail the methods used in the microscopical identification, by color reactions, of chemical groups and compounds as they occur in normal and pathological tissues. Procedures which require costly treatment such as X-ray or ultraviolet spectrography, freeze-drying, etc., have been omitted. The text is divided into ten main sections under the headings: Proteins and their component amino acid groups, Carbohydrates, Lipids, Nucleic acids, Pigments, Enzymes, Miscellaneous methods for organic substances, Inorganic substances, Sundry technical methods, Additional references, and an Appendix.

*Methods of Biochemical Analysis. Vol 7.*

Edited by DAVID GLICK. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. ix + 353 pp. 15 x 23 cm. Price \$9.50.

This volume, the seventh in the series, is the first one in which methods and techniques of biochemical analysis will be reviewed. The contributed chapters cover: Immuno-electrophoretic analysis, Analysis of basic nitrogenous compounds of toxicological importance, Spectrophotometry of translucent biological materials—opal glass transmission method, Determination of inositol, ethanolamine, and serine in lipides, Assay of lipoprotein lipase *in vivo* and *in vitro*, Determination of creatinine and related guanidinium compounds, Determination of ethyl alcohol in blood and tissues, and Determination of heparin. Subject and author indexes for volume 7 and a cumulative index for the series are appended.

*Canadian Cancer Conference. Vol. III.*

Edited by R. W. BEGG. Academic Press Inc., 111 Fifth Ave., New York 3, N. Y., 1959. xiv + 461 pp. 15 x 23 cm. Price \$12.

This volume of reports at the third Conference considers the recently gained knowledge of nucleic acid chemistry. Volume I explored: Experimental tumors, tumor-host relations, enzymes, and ionizing radiations; volume II covered: The cell, leukemia and chemotherapy, hormones and cancer, immunity, and basic mechanisms. The subjects in volume III are grouped under: Nucleic acids, genetics, viruses and tumors, and biology of cancer.

*The Actinomycetes. Vol. I. Nature, Occurrence*

and Activities. By Selman A. Waksman. The Williams and Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1959. xi + 327 pp. 17 x 2.55 cm. Price \$12.50.

This treatise is, in part, a summary of the investigations in soil microbiology during the past 45 years by Dr. Waksman and his associates. It is, without a doubt, an authoritative compilation of the important knowledge about the actinomycetes, which only about 20 years ago were dismissed as a "little-known group of microorganisms." A selected bibliography requiring 41 pages, an index to species of actinomycetes, and a general index are appended.

*Fundamentals of Physical Chemistry.*

By H. I. CROCKFORD and SAMUEL B. KNIGHT. John Wiley & Sons, Inc., 440 Fourth Ave., New York 1 N. Y., 1959. xvii + 463 pp. 14 x 21.5 cm. Price \$6.95.

This book is based upon a 1950 edition entitled "Fundamentals of Physical Chemistry for Pre-medical Students," which was reviewed in THIS JOURNAL, 39, 423 (1950). Prerequisites for this textbook are undergraduate general chemistry, qualitative and quantitative analysis, organic chemistry, and ability to solve quadratic equations and use logarithms. The revised text adds three chapters on thermodynamics, which includes a limited use of calculus. New topics include the change in the value of the equilibrium constant with temperature, the change of vapor pressure with temperature, and the energy of activation in reaction kinetics. The format and style of presentation is similar to that of earlier editions.

*Progress in Drug Research. Vol. I.*

Edited by ERNST JUCKER. Birkhauser Verlag, Basel, Switzerland, 1959. 607 pp. 16.5 x 24 cm. Price sFr. 68.

This is the first volume of a planned series of annual reviews of current activities in fields of pharmaceutical research which include chemical, pharmacological and clinical aspects. Of the seven subjects covered in this volume, five are in German. The two English portions discuss Cholesterol and its relation to atherosclerosis (Lin and Chen of Eli Lilly) and Stereochemical factors in biological activity (A. H. Beckett, Chelsea College School of Pharmacy, London). The placebo problem is discussed in relation to clinical studies with many types of medication. The other subjects covered are: Die Ionenaustauscher und ihre Anwendung in der Pharmazie und Medizin; Die Chemotherapie der Wurmkrankheiten; Neuere Aspekte der chemischen Anthelminticaforschung; and Eine Übersicht der neuen Arzneimittel aus den letzten fünf Jahren.

*The Chemistry of Heterocyclic Compounds. A*

*Series of Monographs. Vol. XIII—s-Triazines and Derivatives.* By EDWARD M. SMOLIN and LORENCE RAPOPORT. Interscience Publishers, 250 Fifth Ave., New York 1, N. Y., 1959. xxiv + 644 pp. 15 x 22.5 cm. Price \$30 single, \$28 subscription.

This is the thirteenth volume to appear of a series of monographs devoted to a comprehensive presentation of heterocyclic chemistry prepared by a group of authorities in this field. The subdivisions have been designed to cover heterocyclic compounds in their entirety by monographs which reflect the importance and interrelationships of the various compounds. This latest volume, in addition to an introductory chapter, covers Cyanuric acid and derivatives, Alkyl(Aryl)-s-triazines; Monohydroxy-, hydroxyamino-, dihydroxy-s-triazines and related compounds; Mono-amino- and diamino-s-triazines; Ammelide, ammeline, and related compounds; Melamine and substituted melamines; Isocyanuric acid and derivatives; Condensed ring s-triazine systems; Hexahydro-s-triazines; Hexamethylenetetramine; and s-Triazaborane and its derivatives.

Scientific Edition

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49

FEBRUARY 1960

NUMBER 2

## Observations on Comparative Growth Studies Between a Streptomycin-Resistant and Streptomycin-Sensitive Strain of *Salmonella schottmuelleri*\*

By RICHARD J. SIMMONS and CHARLES GAINOR

Growth comparisons between a streptomycin-resistant and streptomycin-sensitive strain of *Salmonella schottmuelleri* is reported. The inability of the resistant strain to utilize ammonium citrate, ammonium succinate, *l*-asparagine, aspartic acid, and cystine as sole source of nitrogen stands in sharp contrast to the performance of the sensitive strain. The resistant strain failed to grow in a synthetic medium of mineral salts, glucose, and *l*-asparagine which, however, supported excellent growth of the sensitive strain. Regardless of the nature of the nutritional environments employed in this study, the resistant strain was never stimulated to a level of growth comparable to that of the sensitive strain. Apparently, the rate of utilization of glutamic acid and aspartic acid is the same for each strain, and the two strains produced alanine at approximately the same rate. In biochemical reactions the resistant strain demonstrated a response forty-eight to seventy-two hours later than the sensitive strain, and failed to produce gas from maltose or glucose in seventy-two hours. In contrast, the sensitive strain gave, in twenty-four hours, reactions comparable to a typical *S. schottmuelleri*.

THE PROBLEM of microbial resistance to antibiotics still requires additional information to explain more fully this perplexing phenomenon. Studies were undertaken to ascertain what differences, if any, exist between a streptomycin-resistant and streptomycin-sensitive strain of *Salmonella schottmuelleri* as to relative growth rates, utilization of different carbon and nitrogen sources, and general biochemical reactions.

An excellent article which reviews differences between resistant and sensitive strains of bacteria, in addition to their tolerance of diverse levels of antibiotics, has been published (1).

### EXPERIMENTAL

**Isolation and Maintenance of the Streptomycin-Resistant Strains.**—The parent culture of *Sal-*

*monella schottmuelleri* (ATCC 9282), when subjected to the gradient plate technique (2), was completely inhibited by 10 mcg. per ml. of streptomycin in Difco nutrient agar. Resistant strains of bacteria were those that multiplied equally well in the presence or absence of high concentrations of streptomycin (5,000 mcg. per ml.). Streptomycin-resistant strains were isolated from the parent culture by a single-step selection method (3) and by serial transfers in increasing concentrations of the antibiotic (2). The resistant strain used in all of the subsequent studies could withstand 5,000 mcg. of streptomycin per ml. of medium.

**Preparation of Inocula.**—The preparation of inocula for all experimental studies was carefully standardized to minimize variation in the number of bacteria at the time of inoculation. Cells were carefully removed from eighteen-hour nutrient agar slants to sterile physiological saline. This suspension was centrifuged at 2,000 r. p. m. for thirty minutes, and the process repeated after adding fresh saline to the sedimented cells. The twice-washed cells were then resuspended in fresh saline, and adjusted to the desired absorbance reading. A standard curve relating absorbance readings to numbers of bacteria per ml. of suspension was pre-

\* Received August 18, 1959 from the Department of Microbiology and Public Health, Michigan State University, East Lansing.

This paper is taken in part from a dissertation submitted in partial fulfillment for the degree of Doctor of Philosophy, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pa.

viously constructed for both the streptomycin-resistant and streptomycin-sensitive strains of *S. schottmuelleri*. Unless otherwise stated, 0.1 ml. of bacterial suspension, containing approximately  $8 \times 10^4$  bacteria, was used as the inoculum per 10 ml. of medium.

**Growth Comparisons in Chemically-Defined Media.**—The initial growth studies employed a medium prepared as follows: A basal medium containing 0.2%  $\text{Na}_2\text{SO}_4$ , 0.002%  $\text{MgCl}_2$ , 0.05%  $\text{KH}_2\text{PO}_4$ , 0.15%  $\text{K}_2\text{HPO}_4$ , and 0.2% *l*-asparagine was autoclaved at 120° for ten minutes. Then, 1 ml. of a Seitz-filtered, 2% glucose solution was added aseptically to 9 ml. of basal medium, and all tubes incubated at 37° for twenty-four hours to check sterility. The pH of this medium, without adjustment, was consistently 6.9 to 7.0. It is of particular interest that the streptomycin-resistant strain would not grow in this medium, whereas the sensitive-parent grew rapidly and profusely. Experiments were then conducted to produce, if possible, a stimulation in growth of the resistant strain comparable to that of the sensitive culture by additions of supplements to the synthetic medium.

The nitrogen requirements of the two strains were investigated by replacing the *l*-asparagine of the original medium with various ammonium salts and amino acids. Final concentrations of the amino acids and ammonium salts were 0.005% and 0.1%, respectively.

A study of the carbon requirements was conducted by substituting a number of the dibasic organic acids of the Krebs's cycle for glucose, and by replacing the *l*-asparagine with cysteine-HCl in the original synthetic medium. Final concentrations of the carbon compounds and cysteine-HCl were 0.01%.

The effect of a vitamin mixture and several individual vitamins on the growth of the two strains in the original, as well as other, chemically-defined media was tested. Biotin, nicotinic acid, and pyridoxine-HCl in a final concentration of 2 mcg. per ml. of medium were used.

In critical experiments, growth was measured turbidimetrically with the Bausch and Lomb Spectronic 20 set at 425  $m\mu$  and uninoculated medium used as the reference. Otherwise, the presence or absence of growth was determined by visual inspection of inoculated tubes.

**Amino Acid Utilization.**—Streptomycin-resistant and streptomycin-sensitive strains of *S. schottmuelleri* were inoculated into separate 250-ml. flasks containing 100 ml. of basal medium plus 0.2% glucose, 0.005% *l*-glutamic acid, and 0.005% *l*-aspartic acid. Twice washed, eighteen hour-old cells were used in a final concentration of approximately  $1 \times 10^5$  bacteria per ml. of medium. The two test systems and the uninoculated control were incubated at 37°. At various time intervals during growth the amino acid concentration of the supernatants was determined by quantitative chromatography (4). Separation of the amino acids was carried out for twenty to twenty-four hours in phenol saturated with citrate buffer (5).

**Biochemical Studies.**—All tests were done in triplicate. Seitz-filtered glucose, maltose, sucrose, lactose, mannitol, glycerol, inulin, xylose, cellulose, arabinose, and dextrin in a concentration of 1%

in Difco nutrient broth were used. The IMVIC reactions, gelatin liquefaction,  $\text{H}_2\text{S}$  production, and activity in litmus milk were also observed.

## RESULTS AND DISCUSSION

The streptomycin-sensitive strain of *S. schottmuelleri* gave rise to strains which were highly resistant to streptomycin. Colonies which appeared on plates with 5,000 mcg. per ml. of the antibiotic, when subcultured, proved to consist of highly-resistant bacteria which remained so in the absence of the antibiotic. The resistant strain used throughout this study has remained essentially unchanged after repeated subculture in streptomycin-free media for four months.

The additions of a vitamin mixture and individual vitamins failed to stimulate an increase in bacterial numbers of the resistant strain regardless of the nitrogen or carbon substrate used. Actually, the two strains would grow without these vitamins and were not significantly stimulated by their presence.

The addition of streptomycin (up to 1,000 mcg. per ml.) to either nutrient broth or a chemically defined medium was without effect on the growth of the resistant strain. The resistant strain grew at approximately the same rate and to the same population levels in the presence or absence of streptomycin, thus indicating its independence of the drug.

The inability of the resistant strain to utilize ammonium citrate, ammonium succinate, *l*-asparagine, aspartic acid, cysteine-HCl, and cystine as a sole source of nitrogen, even after seventy-two hours of incubation, stands in sharp contrast to the performance of the sensitive strain (Table I).

The effect of single carbon sources on the growth of the two strains is shown in Table II.

The resistant strain grew on continued subculture in Difco nutrient broth, and in a minimal synthetic medium composed of 0.2%  $\text{Na}_2\text{SO}_4$ , 0.002%

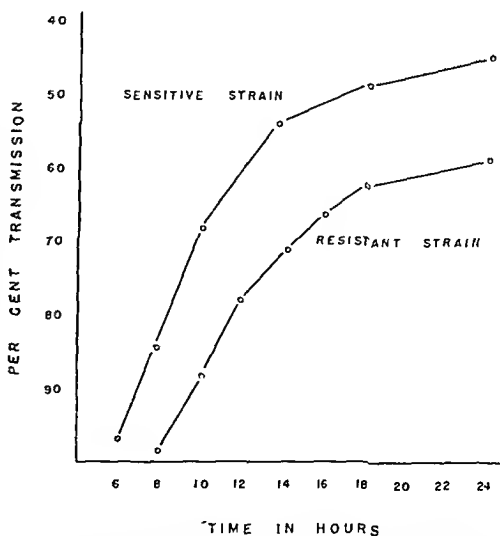


Fig. 1.—Growth curves of streptomycin-resistant and streptomycin-sensitive strains of *S. schottmuelleri* grown in Difco nutrient broth, pH 7.0, at 37°.

TABLE I.—EFFECT OF NITROGEN SOURCE ON GROWTH OF STREPTOMYCIN-RESISTANT AND STREPTOMYCIN-SENSITIVE STRAINS OF *S. schottmuelleri* IN A CHEMICALLY-DEFINED MEDIUM<sup>a</sup>

Nitrogen Source Plus Glucose	Light Transmission, %							
	18	Sensitive, Hours 24	48	72	18	Resistant, Hours 24	48	72
Ammonium chloride <sup>b</sup>	100	100	100	100	100	100	100	100
Ammonium citrate	100	100	90	80	100	100	100	100
Ammonium succinate	100	100	93	83	100	100	100	100
<i>l</i> -Asparagine	100	95	81	74	100	100	100	100
Aspartic acid	97	84	77	70	100	100	100	100
Glutamic acid	98	85	79	72	100	100	96	85
Cysteine-HCl	91	79	72	70	100	100	97	80
Cystine	87	74	66	60	100	100	90	76
Nitrogen Source Minus Glucose								
Ammonium chloride <sup>b</sup>	100	100	100	100	100	100	100	100
Ammonium citrate	100	100	95	88	100	100	100	100
Ammonium succinate	100	100	100	93	100	100	100	100
<i>l</i> -Asparagine	100	100	94	85	100	100	100	100
Aspartic acid	100	100	96	92	100	100	100	100
Glutamic acid	100	96	88	80	100	100	100	95
Cysteine-HCl	100	100	100	100	100	100	100	100
Cystine	100	100	100	93	100	100	100	100

<sup>a</sup> Medium contains 0.2% Na<sub>2</sub>SO<sub>4</sub>, 0.002% MgCl<sub>2</sub>, 0.05% KH<sub>2</sub>PO<sub>4</sub>, 0.15% K<sub>2</sub>HPO<sub>4</sub>, and 0.2% glucose, where indicated.  
<sup>b</sup> Same results also apply to ammonium nitrate, ammonium phosphate, and methionine 100, no growth; 99-90, poor growth; 89-80, fair growth; 79-70, good growth, and 69-60, excellent growth.

TABLE II.—EFFECT OF CARBON SOURCE ON GROWTH OF STREPTOMYCIN-RESISTANT AND STREPTOMYCIN-SENSITIVE STRAINS OF *S. schottmuelleri* IN A CHEMICALLY-DEFINED MEDIUM<sup>a</sup>

Carbon Source	Light Transmission, %							
	18	Sensitive, Hours 24	48	72	18	Resistant, Hours 24	48	72
Citric acid	100	92	84	77	100	100	100	100
Fumaric acid	100	100	91	85	100	100	100	100
Malic acid	100	93	89	83	100	100	100	93
Oxaloacetic acid	100	95	91	87	100	100	100	92
$\alpha$ -Ketoglutaric acid	100	97	94	92	100	100	100	98
Glucose	92	83	79	73	100	100	92	84
Succinic acid	100	94	90	87	100	100	96	89
<i>cis</i> -Aconitic acid	100	100	92	88	100	100	100	93
Cysteine (control) <sup>b</sup>	100	100	100	100	100	100	100	100

<sup>a</sup> Medium contains 0.2% Na<sub>2</sub>SO<sub>4</sub>, 0.002% MgCl<sub>2</sub>, 0.05% KH<sub>2</sub>PO<sub>4</sub>, 0.15% K<sub>2</sub>HPO<sub>4</sub>, and 0.01% cysteine-HCl.  
<sup>b</sup> Cysteine furnished the nitrogen for all tubes, note that cysteine cannot furnish both the carbon and nitrogen sources when used alone. 100, no growth; 99-90, poor growth; 89-80, fair growth; 79-70, good growth; and 69-60, excellent growth.

MgCl<sub>2</sub>, 0.05% KH<sub>2</sub>PO<sub>4</sub>, 0.2% glucose, and 0.005% *l*-glutamic acid. However, growth was sparse in the latter medium and not suitable for observing an increase in bacterial numbers turbidimetrically. Adding 0.005% concentrations of *l*-aspartic acid and cystine to this medium afforded a suitable test system for this purpose. The relative growth rates of the two strains in nutrient broth and in this latter synthetic medium are given in Figs. 1 and 2, respectively.

It was important to note that, regardless of the nature of the nutritional environment employed in this study, the resistant strain was never stimulated to a level of growth comparable to that of the sensitive strain. This growth lag displayed by the resistant variant is an interesting variational character similar to the slower growth rates described for certain phage-resistant variants (6), and for streptomycin-resistant *Salmonella* (7).

No differences were observed in utilization of glutamic acid and aspartic acid by the two strains, although the resistant strain took twenty to twenty-four hours longer to effect the same degree of utilization as did the sensitive strain. Apparently, the rate of utilization of these two amino acids was commensurate with the relative growth rates

of each strain. For a given strain, plots of time versus viable counts, and plots of time versus amino acid utilization would yield identical curves.

During the amino acid studies, the appearance of a new ninhydrin positive spot on the chromatograms was observed. Subsequent studies proved this new spot to be alanine. Although no attempt was made to quantitate alanine production by the two strains, visual comparisons of spot size, color intensity, and disappearance of glutamate and aspartate were significant in determining whether differences in alanine production existed between the two strains. These observations indicated that the two strains produced alanine at about the same rate, when glutamate and aspartate were used as the substrate.

It was interesting to note that the streptomycin-resistant *S. schottmuelleri* failed to grow in Koser's citrate medium. Growth could not be initiated whether  $1 \times 10^4$  cells or  $1 \times 10^6$  washed cells per ml. of medium was used. However, the streptomycin-sensitive *S. schottmuelleri* grew rapidly and profusely in the same medium when only  $1 \times 10^4$  washed cells per ml. of medium was used, and the strain could be successfully subcultured in the medium.

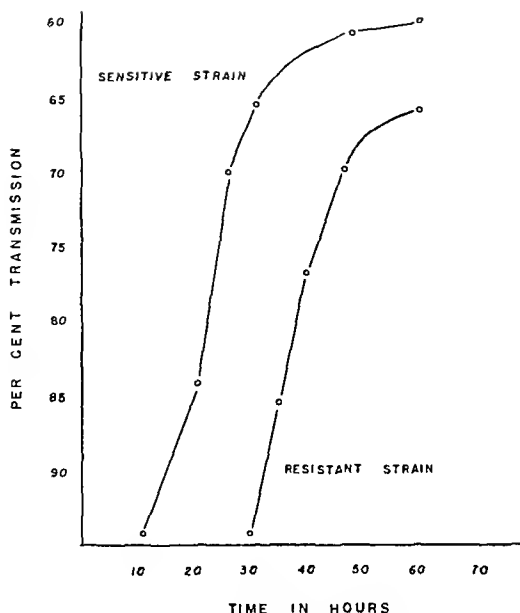


Fig. 2.—Growth curves of streptomycin-sensitive and streptomycin-resistant strains of *S. schottmuelleri* grown in a chemically-defined medium, pH 7.0, at 37°. Medium composed of 0.2%  $\text{Na}_2\text{SO}_4$ , 0.002%  $\text{MgCl}_2$ , 0.05%  $\text{KH}_2\text{PO}_4$ , 0.2% glucose, 0.005% *l*-glutamic acid, 0.005% *l*-aspartic acid, and 0.005% cystine.

In biochemical reactions the resistant strain demonstrated a response forty-eight to seventy-two hours later than the sensitive strain and failed to produce gas from maltose or glucose in seventy-two hours. In contrast, the sensitive strain gave, in twenty-four hours, reactions comparable to those noted in Bergey's Manual (8) for a typical *S. schottmuelleri*. Seligmann and Wassermann (7) reported that their streptomycin-resistant strains of *Salmonella* completely lost the ability to produce gas from carbohydrates, whereas the resistant strain used in this study lost the ability to produce gas only from maltose or glucose among the carbohydrates tested.

### SUMMARY

Comparisons of a streptomycin-resistant and a streptomycin-sensitive strain of *S. schottmuelleri* as to differences in relative growth rates, utilization of different carbon and nitrogen sources, and biochemical reactions have been conducted.

The parent strain of *S. schottmuelleri* (ATCC 9282) gave rise to strains which were highly re-

sistant to streptomycin. Resistance to this drug was found to be a relatively stable characteristic. A strain resistant to 5,000 mcg. of streptomycin per ml. of medium has remained resistant to this concentration after four months of repeated subculture in streptomycin-free media.

In general, the two strains displayed qualitative biochemical reactions comparable to those noted in Bergey's Manual (8) for a typical *S. schottmuelleri*. However, in contrast to the parent strain, the resistant strain would not grow in Koser's citrate medium, failed to produce gas from maltose or glucose in seventy-two hours, and consistently exhibited a forty-eight to seventy-two-hour delay in biochemical reactions.

A significant difference in growth was detected between the streptomycin-resistant and streptomycin-sensitive strains. The resistant variant failed to grow in a chemically-defined medium consisting of mineral salts, glucose, and *l*-asparagine which, however, supported growth of the sensitive strain. Regardless of the nature of the nutritional environments employed in this study, the resistant strain was never stimulated to a level of growth comparable to that of the sensitive strain. The resistant variant grew at approximately the same rate in the presence or absence of streptomycin.

No differences were observed in utilization of glutamic acid and aspartic acid by the two strains. Apparently, the rate of utilization of these two amino acids was commensurate with the relative growth rates of each strain. On the basis of qualitative studies, it was believed that alanine was produced at the same rate by both strains.

### REFERENCES

- (1) Eagle, H., and Saz, A. K., *Ann. Rev. Microbiol.*, 9, 173(1955).
- (2) Szybalski, W., and Bryson, V., *J. Bacteriol.*, 64, 489 (1952).
- (3) Klein, M., and Kimmelman, L. J., *ibid.*, S2, 471(1946).
- (4) Housewright, R. D., and Thorne, C. B., *ibid.*, 60, 89 (1950).
- (5) Berry, H. K., and Cain, L., *Arch. Biochem.*, 24, 179 (1949).
- (6) Luria, S. E., *Cold Spring Harbor Symposia Quant. Biol.*, 11, 130(1946).
- (7) Seligmann, E., and Wassermann, M., *J. Immunology*, 57, 351(1947).
- (8) Breed, R. S., Murray, E. G. D., and Smith, N. R., "Bergey's Manual of Determinative Bacteriology," 7th ed., The Williams and Wilkins Co., Baltimore, 1957, pp. 373, 374.

# The Effect of Granule Size Upon Disintegration Time and Capping in Compressed Tablets\*

By ALBERT J. FORLANO† and LEONARD CHAVKIN

The effect of compressing several mesh sizes of lactose, sodium bicarbonate, and magnesium trisilicate granulations into tablets was determined. A definite relationship was found to exist between the particle size of the granulation of a particular substance and the disintegration rate, per cent of capping, and other physical tablet characteristics. A definition of the term fines was also proposed.

THE PARTICLE SIZE of the granulated material is an important determinant of the characteristics of the finished compressed tablet. The purpose of this investigation was to determine the effect of compressing various mesh sizes of lactose, sodium bicarbonate, and magnesium trisilicate granulations into tablets, separately. The effect of granule size upon the following characteristics were determined: (a) disintegration rate, (b) frequency of capping, and (c) production problems associated with each mesh size.

The determination of a definition for the commonly used term, fines, and the respective proportion of each mesh size in the granulations were also considered pertinent to this study.

## EXPERIMENTAL

Sodium bicarbonate, lactose, and magnesium trisilicate were chosen as the materials in this study because they represent varying degrees of solubility, and will be referred to as the "active ingredients."

A preliminary study was conducted by granulating the three active ingredients with 10% acacia, 50% glucose, and syrup-alcohol (equal parts), in order to determine the most suitable binders. Ten per cent acacia in water was found to be suitable for lactose and sodium bicarbonate and 50% syrup U. S. P.-alcohol was chosen for magnesium trisilicate.

**Procedure.**—The three active ingredients and corn starch were dried in an oven at 120° F. for three days prior to use. After drying, fifteen pounds of each active ingredient was mixed with 2.65 pounds of corn starch (the active ingredient represented 85% of the mixture and the corn starch 15%). This mixture was passed twice through a Fitzpatrick comminuting machine fitted with a 120-mesh screen. The resultant material passed through a 200-mesh screen.

The powder mixture was placed in a Stokes mixer model 21-H, mixed for one hour, and subsequently

granulated with the respective binders. The wet material was passed through a Stokes oscillating granulator model 43-A, fitted with a No. 6-mesh screen, and dried at 120° F. for one week. The dried material was rescreened.

The dried granulation was fractionated by the use of a mechanical shaker and a set of U. S. Bureau of Standard sieves. The following fractions were collected: a control consisting of the original granulation, 6-8, 8-10, 10-12, 12-16, 16-20, 20-30, 30-40, 40-60, 60-80, 80-100, 100-140, 140-200, and 200 mesh and finer (Table I). The fractions were lubricated with 1% of calcium stearate.

TABLE I.—PERCENTAGE OF EACH MESH FRACTION BASED ON THE TOTAL WEIGHT OF GRANULATION\*

Mesh Size	Magnesium Trisilicate	Sodium Bicarbonate	Lactose
6-8	2.6	..	0.5
8-10	1.6	8.8	5.5
10-12	5.3	8.1	6.7
12-16	17.1	16.6	14.8
16-20	20.8	13.9	13.6
20-30	16.3	12.6	11.7
30-40	19.3	11.1	9.9
40-60	8.6	10.3	13.0
60-80	2.6	4.4	7.3
80-100	1.1	1.9	4.3
100-140	1.0	2.4	4.9
140-200	0.7	1.6	2.5
200 and finer	3.0	8.4	5.9

\* Five hundred grams of the original granulations were removed before fractionation and used as a control.

Each granulation fraction was compressed on a Stokes model B-2 rotary tablet machine. The pressure was controlled by the use of the safety mechanism, which is essentially a spring attached to the lower pressure wheel. This spring was set to release upon receiving more than four tons of pressure, therefore if that pressure was exceeded, any pressure in excess of four tons would be lost due to the release of the spring. The tablets were compressed with the spring constantly releasing itself. The compression time for each fraction was set at forty to fifty seconds to minimize the possibility of spring fatigue.

One set of 13/32 inch standard concave punches and dies was used for the compression. After the machine was set up it was allowed to operate, and the pressure was slowly increased until the noise indicating that the safety spring was releasing pressure in excess of four tons was just perceptible. The tablets were compressed under these conditions.

## RESULTS

**Disintegration Rates.**—Six tablets were chosen represented by a weight of  $0.50 \pm 0.02$  Gm. and were subjected to U. S. P. disintegration tests (1).

\* Received June 30, 1958 from the Ohio State University, Columbus 10.

† This paper was a recipient of the Lunsford Richardson Pharmacy Award in Eastern United States in 1958.

Abstracted from a thesis presented to the College of Pharmacy, Columbia University, by Albert J. Forlano in partial fulfillment of the requirements for the degree of Master of Science.

† Present Address: Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

The disintegration tests on magnesium trisilicate tablets showed that (with the exception of the 200-mesh and finer fraction) they would not disintegrate completely and left a small, flat, hard core. Therefore, the end point for these tablets was the flat, hard core stage. A plot of disintegration time versus mesh size are found in Figs 1 and 2

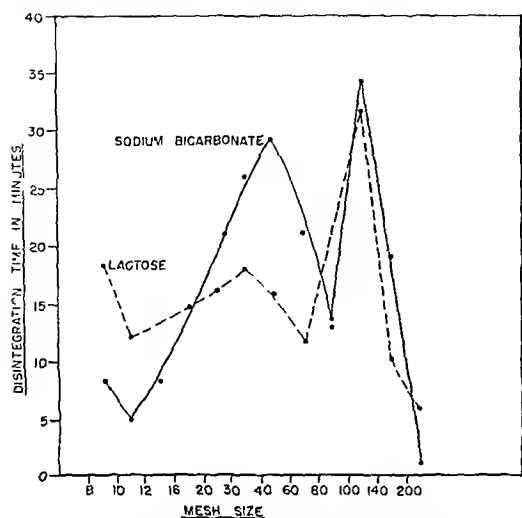


Fig 1—Disintegration time for tablets of sodium bicarbonate and lactose of various mesh sizes

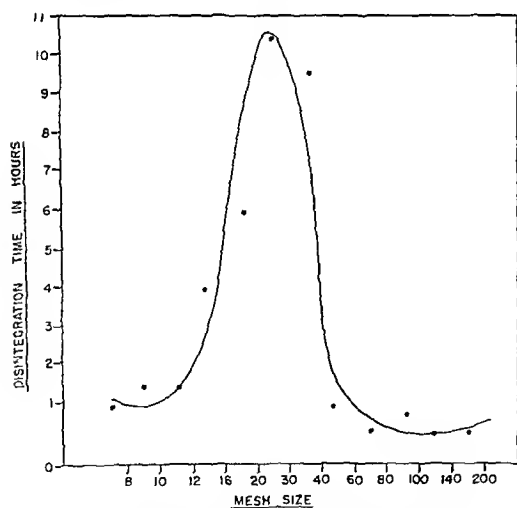


Fig. 2—Disintegration time for tablets of magnesium trisilicate of various mesh sizes

The average U S P. disintegration rates of two runs were used in plotting the data. These values are given in Table II

**Per Cent of Capping.**—This was determined by placing a jar containing the tablets into a mechanical shaker and allowing them to shake for five minutes. Then the contents of the jars were weighed and the amount of capped material was determined by weighing the amount that would pass through a No. 10 screen. The per cent of capping was determined by Eq 1. This information is presented in Fig. 3.

TABLE II—U S P DISINTEGRATION RATES FOR SODIUM BICARBONATE, LACTOSE, AND MAGNESIUM TRISILICATE TABLETS

Mesh Size	Sodium Bicarbonate, min	Lactose, min	Magnesium Trisilicate, hr
8-10	8.5	19.5	0.81
10-12	5.0	12.5	1.40
12-16	8.2	13.0	3.80
16-20	14.5	14.5	6.00
20-30	21.0	16.0	10.50
30-40	26.0	18.0	9.58
40-60	29.0	16.0	0.91
60-80	21.0	11.5	0.61
80-100	14.0	13.5	0.71
100-140	34.0	31.5	0.53
140-200	19.0	10.2	0.40
200 mesh and fines	1 <sup>a</sup>	6.0	0.32

<sup>a</sup> These tablets crumbled upon ejection

$$\frac{\text{Weight of capped material}}{\text{Weight of entire sample}} \times 100 = \text{Per cent capping (Eq 1)}$$

**Per Cent of Each Size in Total Granulation.**—The distribution of the different mesh sizes in the granulations were recorded and tabulated in Table I. These results are not to be construed as showing a general pattern because they can be affected by many variables present in the granulation of a tablet mass

## DISCUSSION

It was found, generally, that tablets made from granules larger than 16 mesh, possessed a veined structure. This was probably due to the lack of fine particles to fill the intergranular spaces. The particles of 200 mesh and finer of soluble materials

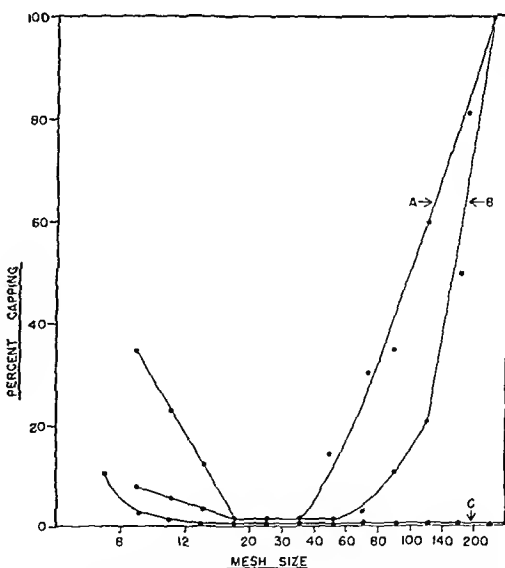


Fig 3—Per cent capping found among tablets of various mesh sizes of lactose, sodium bicarbonate, and magnesium trisilicate (respectively, A, B, and C).

did not form tablets upon compression, but resulted in capped masses.

**Sodium Bicarbonate.**—The incidence of capping was least in the range of 16–40 mesh; however, capping increased with decreasing mesh size following 60 mesh. This increased capping might be explained by the fact that granules, finer than 60 mesh, were not as well granulated as the larger granules, and under compression their granule structure was crushed resulting in a loss of cohesion. No tablets could be made using 200-mesh and finer granules.

The disintegration data showed that tablets made, using granules of 16–60 mesh, had the longest disintegration times.

As the granule size decreased below 60 mesh the disintegration time decreased with an unexpected rise at 100–140 mesh, followed by a decrease at 140–200 mesh (Fig. 3). An increase in capping was also exhibited in the 60 to 200 mesh and finer range. Tablets made from granules smaller than 60 mesh lost the glossy appearance that was characteristic of the larger mesh sizes.

**Lactose.**—The behavior of the lactose granulations closely followed the pattern exhibited by sodium bicarbonate granulations. However, there was a minor difference; disintegration time and per cent of capping indicated the lactose granules were becoming weaker at levels smaller than 40 mesh. The comparable change with sodium bicarbonate occurred at 60 mesh.

**Magnesium Trisilicate.**—The tablets made using 12 to 30-mesh granules had longer disintegration times than the smaller mesh sizes. When tablets were made from granules smaller than 30 mesh the disintegration time decreased, and per cent of capping was negligible.

Examination of the disintegration rates shows that the disintegration time decreased from ten hours beginning with 30–40 mesh, to nineteen minutes at 200 mesh and finer. Only the tablets made from 200 mesh and finer granules were found to disintegrate completely; in all other cases the tablets left a small, hard core. The core might be explained by Johnston (2) who stated that the center of a compressed body consists of a matted, hard core. This core formation, accentuated by the insoluble nature of the material, probably prevented the water from penetrating into the center of the tablet to effect complete disintegration.

The decrease in disintegration time observed as the smaller particles were compressed was opposite to the trends observed with the other granulations of this study.

It is possible that the nature of the bonding occurring either in the granules or in forming the lactose and sodium bicarbonate tablets was different from that in the magnesium trisilicate tablets. It is also conceivable that the effects observed may have been due to the different actions of acacia and sucrose as binders. However, from the data collected in this investigation it was not possible to offer an adequate explanation for the anomalous behavior of the magnesium trisilicate granulations.

**Fines.**—As a result of this study it was possible to formulate a definition of fines for the particular substances tested here. Sodium bicarbonate and lactose showed that the disintegration time decreased, binding of punches and dies increased, and per cent of capping increased in the vicinity of 40 to 80 mesh. Since an excess of these particles and smaller ones were detrimental to the compression of satisfactory tablets they may be considered fines.

In the compression of the magnesium trisilicate tablets, particles smaller than 60–80 mesh caused binding between the punches and the dies and were therefore considered fines.

## SUMMARY

1. The relationship between the particle size of the granulated material and disintegration time and capping of resultant tablets was demonstrated.

2. Sodium Bicarbonate.—Tablets compressed using granules in the range of 8 to 40 mesh exhibited a rise in disintegration time and capping decreased as 60 mesh was approached. With tablets compressed using granules of 60 mesh and smaller, the disintegration time decreased and capping increased; this trend continued through 200 mesh and finer, at which point tablets could no longer be made. The optimum granule size for compression of sodium bicarbonate tablets appeared to be 16 to 60 mesh.

3. Lactose.—These tablets exhibited the same characteristics as sodium bicarbonate tablets except that the transition to poorer tablets occurred after 40 mesh. Consequently, the optimum particle size for lactose granulations was found to be in the 16–40 mesh range.

4. Magnesium Trisilicate.—The per cent capping decreased as the mesh size went from 16 to 200 mesh and finer. At 200 mesh and finer the per cent of capping was smallest. From 30–200 mesh and finer the disintegration time decreased. Due to binding of dies and punches with sizes below 60 mesh, the best mesh sizes for these tablets were 16 to 60 mesh.

5. Fines.—Granules of 40–60 mesh and smaller were considered fines in the sodium bicarbonate and lactose granulations; granules of 60 mesh or smaller can be considered fines in the magnesium trisilicate granulations.

## REFERENCES

- (1) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1950, p. 700.
- (2) Johnston, J., and Adams, L. H., *J. Am. Chem. Soc.*, **34**, 563(1912).



The disintegration tests on magnesium trisilicate tablets showed that (with the exception of the 200 mesh and finer fraction) they would not disintegrate completely and left a small, flat, hard core. Therefore, the end point for these tablets was the flat, hard core stage. A plot of disintegration time versus mesh size are found in Figs 1 and 2.

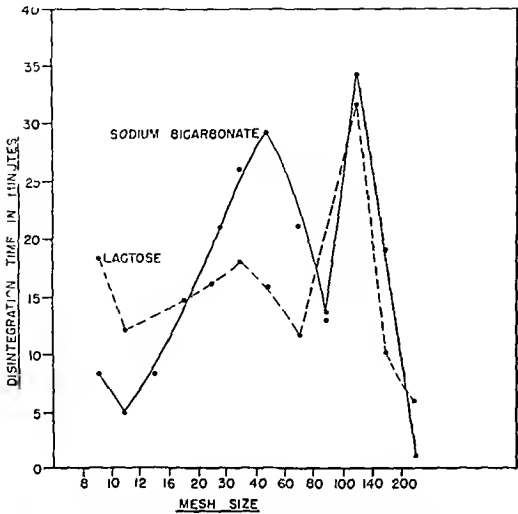


Fig 1—Disintegration time for tablets of sodium bicarbonate and lactose of various mesh sizes

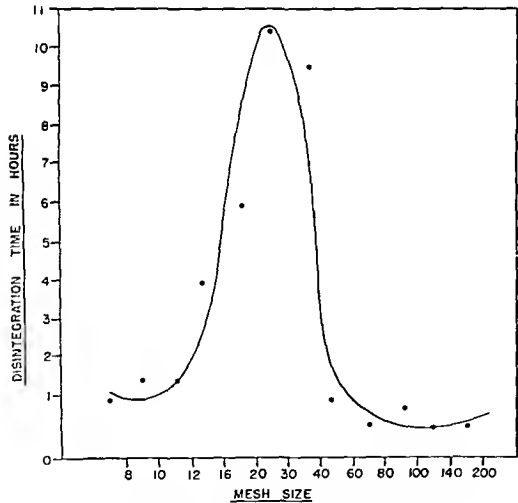


Fig 2—Disintegration time for tablets of magnesium trisilicate of various mesh sizes

The average U S P disintegration rates of two runs were used in plotting the data. These values are given in Table II.

**Per Cent of Capping.**—This was determined by placing a jar containing the tablets into a mechanical shaker and allowing them to shake for five minutes. Then the contents of the jars were weighed and the amount of capped material was determined by weighing the amount that would pass through a No. 10 screen. The per cent of capping was determined by Eq 1. This information is presented in Fig 3.

TABLE II—U S P DISINTEGRATION RATES FOR SODIUM BICARBONATE, LACTOSE, AND MAGNESIUM TRISILICATE TABLETS

Mesh Size	Sodium Bicarbonate, min	Lactose min	Magnesium Trisilicate hr
8-10	8 5	19 5	0 81
10-12	5 0	12 5	1 40
12-16	8 2	13 0	3 80
16-20	14 5	14 5	6 00
20-30	21 0	16 0	10 50
30-40	26 0	18 0	9 58
40-60	29 0	16 0	0 91
60-80	21 0	11 5	0 61
80-100	14 0	13 5	0 71
100-140	34 0	31 5	0 53
140-200	19 0	10 2	0 40
200 mesh and fines	1 <sup>a</sup>	6 0	0 32

<sup>a</sup> These tablets crumbled upon ejection

$$\frac{\text{Weight of capped material}}{\text{Weight of entire sample}} \times 100 = \text{Per cent capping (Eq 1)}$$

**Per Cent of Each Size in Total Granulation.**—The distribution of the different mesh sizes in the granulations were recorded and tabulated in Table I. These results are not to be construed as showing a general pattern because they can be affected by many variables present in the granulation of a tablet mass.

DISCUSSION

It was found, generally, that tablets made from granules larger than 16 mesh, possessed a vemed structure. This was probably due to the lack of fine particles to fill the intergranular spaces. The particles of 200 mesh and finer of soluble materials

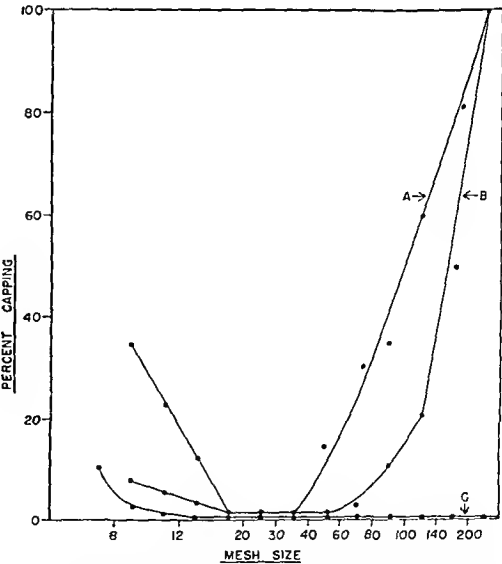


Fig 3—Per cent capping found among tablets of various mesh sizes of lactose, sodium bicarbonate, and magnesium trisilicate (respectively, A, B, and C).

did not form tablets upon compression, but resulted in capped masses.

**Sodium Bicarbonate.**—The incidence of capping was least in the range of 16–40 mesh; however, capping increased with decreasing mesh size following 60 mesh. This increased capping might be explained by the fact that granules, finer than 60 mesh, were not as well granulated as the larger granules, and under compression their granule structure was crushed resulting in a loss of cohesion. No tablets could be made using 200-mesh and finer granules.

The disintegration data showed that tablets made, using granules of 16–60 mesh, had the longest disintegration times.

As the granule size decreased below 60 mesh the disintegration time decreased with an unexpected rise at 100–140 mesh, followed by a decrease at 140–200 mesh (Fig. 3). An increase in capping was also exhibited in the 60 to 200 mesh and finer range. Tablets made from granules smaller than 60 mesh lost the glossy appearance that was characteristic of the larger mesh sizes.

**Lactose.**—The behavior of the lactose granulations closely followed the pattern exhibited by sodium bicarbonate granulations. However, there was a minor difference; disintegration time and per cent of capping indicated the lactose granules were becoming weaker at levels smaller than 40 mesh. The comparable change with sodium bicarbonate occurred at 60 mesh.

**Magnesium Trisilicate.**—The tablets made using 12 to 30-mesh granules had longer disintegration times than the smaller mesh sizes. When tablets were made from granules smaller than 30 mesh the disintegration time decreased, and per cent of capping was negligible.

Examination of the disintegration rates shows that the disintegration time decreased from ten hours beginning with 30–40 mesh, to nineteen minutes at 200 mesh and finer. Only the tablets made from 200 mesh and finer granules were found to disintegrate completely; in all other cases the tablets left a small, hard core. The core might be explained by Johnston (2) who stated that the center of a compressed body consists of a matted, hard core. This core formation, accentuated by the insoluble nature of the material, probably prevented the water from penetrating into the center of the tablet to effect complete disintegration.

The decrease in disintegration time observed as the smaller particles were compressed was opposite to the trends observed with the other granulations of this study.

It is possible that the nature of the bonding occurring either in the granules or in forming the lactose and sodium bicarbonate tablets was different from that in the magnesium trisilicate tablets. It is also conceivable that the effects observed may have been due to the different actions of acacia and sucrose as binders. However, from the data collected in this investigation it was not possible to offer an adequate explanation for the anomalous behavior of the magnesium trisilicate granulations.

**Fines.**—As a result of this study it was possible to formulate a definition of fines for the particular substances tested here. Sodium bicarbonate and lactose showed that the disintegration time decreased, binding of punches and dies increased, and per cent of capping increased in the vicinity of 40 to 80 mesh. Since an excess of these particles and smaller ones were detrimental to the compression of satisfactory tablets they may be considered fines.

In the compression of the magnesium trisilicate tablets, particles smaller than 60–80 mesh caused binding between the punches and the dies and were therefore considered fines.

## SUMMARY

1. The relationship between the particle size of the granulated material and disintegration time and capping of resultant tablets was demonstrated.

2. Sodium Bicarbonate.—Tablets compressed using granules in the range of 8 to 40 mesh exhibited a rise in disintegration time and capping decreased as 60 mesh was approached. With tablets compressed using granules of 60 mesh and smaller, the disintegration time decreased and capping increased; this trend continued through 200 mesh and finer, at which point tablets could no longer be made. The optimum granule size for compression of sodium bicarbonate tablets appeared to be 16 to 60 mesh.

3. Lactose.—These tablets exhibited the same characteristics as sodium bicarbonate tablets except that the transition to poorer tablets occurred after 40 mesh. Consequently, the optimum particle size for lactose granulations was found to be in the 16–40 mesh range.

4. Magnesium Trisilicate.—The per cent capping decreased as the mesh size went from 16 to 200 mesh and finer. At 200 mesh and finer the per cent of capping was smallest. From 30–200 mesh and finer the disintegration time decreased. Due to binding of dies and punches with sizes below 60 mesh, the best mesh sizes for these tablets were 16 to 60 mesh.

5. Fines.—Granules of 40–60 mesh and smaller were considered fines in the sodium bicarbonate and lactose granulations; granules of 60 mesh or smaller can be considered fines in the magnesium trisilicate granulations.

## REFERENCES

- (1) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1950, p. 700.
- (2) Johnston, J., and Adams, L. H., *J. Am. Chem. Soc.*, **34**, 563(1912).

# Pressurized Pharmaceutical Aerosols for Inhalation Therapy I\*

## Physical Testing Methods

By IRVING PORUSH, CHARLES G. THIEL, and JAMES G. YOUNG

The physical testing methods used for controlling the manufacture of pressurized aerosols for inhalation therapy are described. Results of the tests for uniformity of valve delivery, particle size, and pressure of this new pharmaceutical dosage form are reported. A reliable and reproducible aerosolized dose of medication can be administered for inhalation therapy by means of the metering valve on the pressurized container.

THE INTRODUCTION of pressurized aerosols for inhalation therapy provided a new pharmaceutical dosage form for dispensing a measured quantity of medication in the precise particle size required for optimum results. Clinical evaluation of the effectiveness and acceptability by users of this form of medication are reported in the literature (1-6). A number of advantages of packaging in pressurized containers are described by Sciarra (7), Root (8), and Barr (9). Among the more compelling ones are protection of contents from atmospheric oxidation, moisture, and bacterial contamination; and accurate delivery of a measured dose of aerosolized drug. The reliability of dispensing accurate dosage by this means, as with other pharmaceuticals, is largely dependent upon the controls and specifications imposed on its manufacture. Physical measurements of particle size, pressure, and uniformity of valve delivery are new criteria for efficacy that must be rigorously controlled to achieve a dependable pharmaceutical preparation. A description of the methods and techniques employed in the physical testing of Medihaler<sup>1</sup> pressurized aerosols is presented here.

### METHODS AND TECHNIQUES

**Particle Size Analysis.**—The efficacy of inhalation aerosols is as dependent upon particle size as on the concentration of medicament. There is general agreement in the literature that optimum particle diameter for penetration into the pulmonary depths is between one-half and seven  $\mu$  (10-12). Smaller particles are exhaled and larger ones do not penetrate to the desired depth. Determination of particle di-

mensions must be accurate, reproducible, and sufficiently rapid for routine application.

Methods employed in the analysis of particles from pressurized containers are dependent, to some extent, on whether they are solutions or suspensions in propellant. The solution of drugs in fluorohydrocarbons usually requires the use of a cosolvent (e. g., alcohol), in about 50% of the volume. Sprays of these solutions take several seconds for the higher boiling cosolvent to evaporate, and the particles change dimensions as vaporization occurs. Any measurement of these particles in this dynamic system must take into account the rate of evaporation and correlate this rate with the time necessary for inspiration of medication.

The Cascade impactor described by Pilcher, *et al* (13), and Tarpley (14) is most suitable for measurement of the particle size of solution-type aerosols. This method depends on the principle that in an air-borne stream of particles projected through a series of nozzles and glass slides at increasing velocity larger particles are impacted first on the lower velocity stages, while smaller particles pass on and are collected on later stages. The most serious deficiency of this apparatus is the relatively large sample needed for analysis. This makes determinations of particles emanating from single doses of most aerosols impossible by ordinary analytical methods. Measurements of particle size were made with the Cascade impactor and the distribution determined on an isoproterenol aerosol solution containing 0.25% w/w isoproterenol HCl in a propellant with 35% w/w (50% v/v) alcohol. The mass median diameter was 7  $\mu$ ; 45% of the mass was less than 5, 50% less than 7, and 58% less than 10  $\mu$ .

Preparations, such as the Medihaler, containing suspensions of micronized drug in liquefied propellant without a cosolvent, present a different problem in particle size analysis. The boiling point of the propellant mixture is about  $-17^{\circ}$ , resulting in almost instantaneous vaporization on reaching atmospheric pressure, and leaving the solid particles suspended in the air-gas stream. Measurement of these particles can be made more readily by use of almost any sizing apparatus, and more important, can be checked by microscopic observation. A rapid, reproducible apparatus for measurement of small particle size distribution is described by Dimmick, *et al.* (15). The technique used is termed "the light scatter decay method," and involves a simplified analysis of the change in light intensity of a Tyndall beam as an aerosol settles under turbulent conditions. Only one or two measured doses of the aerosol are required, making possible its application to the study of stability of particle dimensions on prolonged storage.

The distribution of particles is usually reported by giving the mass median diameter as found in the light

\* Received August 21, 1959, from the Riker Laboratories, Inc., Northridge, Calif.

<sup>1</sup> Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

<sup>1</sup> Medihaler-Iso (Riker) is a suspension of 2 mg./cc. isoproterenol sulfate in an inert propellant. Medihaler-Epi (Riker) is a suspension of 7 mg./cc. epinephrine bitartrate in an inert propellant.

scatter decay apparatus. In addition, it is of interest to determine the per cent of the mass of the material in particles less than 5  $\mu$ , less than 7  $\mu$ , and less than 10  $\mu$ . The specification established in this laboratory for material acceptable for use in inhalation therapy is "the mass median diameter determined on the light scatter decay apparatus is 4  $\mu$  or less, and 85% or more of the mass is less than 7  $\mu$  in diameter." Typical analyses of recent runs of Medihaler-Iso and Medihaler-Epi aerosols are shown in Table I.

TABLE I.—PARTICLE SIZE OF ISOPROTERENOL AND EPINEPHRINE AEROSOL SUSPENSIONS

Lot No.	Mass Median Diameter, $\mu$	Mass of Particles with Diameters		
		<5 $\mu$ , %	<7 $\mu$ , %	<10 $\mu$ , %
ISO-91782	3.5	70	88	98
ISO-91795	2.7	78	92	99
ISO-91794	2.9	77	92	99
EPI-91797	3.5	73	93	100

An important consideration in the manufacture of pressurized pharmaceutical aerosol suspensions is the stability of particle dimensions on prolonged storage. To demonstrate that growth of the microscopic particles does not occur in Medihaler-Iso and Medihaler-Epi, their particle size was determined at various intervals after manufacture on the light scatter decay apparatus. Results are shown in Table II.

TABLE II.—STABILITY OF PARTICLE DIMENSIONS OF MEDIHALER-ISO AND MEDIHALER-EPI

Lot No.	Storage Period, 22°	Mass Median Diameter, $\mu$	Mass of Particles with Diameters		
			<5 $\mu$ , %	<7 $\mu$ , %	<10 $\mu$ , %
ISO-X9012	Initial	2.5	79	94	98
ISO-X9012	3 months	2.7	82	95	99
ISO-X9012	6 months	2.5	83	96	99
ISO-71240	18 months	3.0	77	92	99
EPI-71288	18 months	3.2	75	92	99

**Valve Delivery.**—Uniformity of dose from the pressurized aerosol is dependent upon the reproducibility of the valve delivery. Inasmuch as this is a mechanical device, and the quantity delivered is only 50  $\mu$ L. per depression, very fine tolerances must be set in production of the valve components to achieve good reproducibility.

Dosage is controlled by taking a statistical sample of every finished lot of valves and testing for delivery. The weight before and after a single valve depression is recorded and converted to volume by calculation from the known density of the suspension. Variability of dose from an individual valve and from valve to valve is very small. Measurements of recent lots of Medihaler-Iso and Medihaler-Epi aerosols are shown in Tables III and IV.

Specifications for acceptance of Medihaler vials include tolerances for valve delivery as determined on the finished product. All valves must deliver between 85 and 115% of the calculated 50  $\mu$ L. dosage.

**Pressure.**—Particle size is related in varying degrees to the internal pressure of both solution- and suspension-type aerosols. For solutions, the wetness of spray and particle size are almost completely dependent upon internal pressure. Temperature and pressure changes have a profound effect on the dis-

TABLE III.—VALVE TO VALVE REPRODUCIBILITY OF DELIVERY

Lot	No. Vials Tested	Mean Delivery, $\mu$ L.	Stand-	Range, $\mu$ L.
			ard Deviation, $\mu$ L.	
EPI-91798	50	48.1	$\pm 1.6$	44.4–53.2
EPI-91797	24	49.8	$\pm 1.3$	46.5–52.1
ISO-91796	24	51.2	$\pm 1.0$	47.8–53.6
ISO-91795	24	50.4	$\pm 1.4$	46.7–52.3
ISO-91794	24	49.4	$\pm 1.8$	46.1–52.9
ISO-91753	100	51.2	$\pm 1.4$	45.8–54.3

TABLE IV.—REPRODUCIBILITY OF INDIVIDUAL VALVES

Volume Delivered by Valve at 20-Shot Intervals Until Contents of Vial Are Emptied

	Mean Delivery, $\mu$ L.	Standard Deviation, $\mu$ L.	Range, $\mu$ L.
Valve 1	48.4	$\pm 0.35$	48.0–49.0
Valve 2	49.4	$\pm 0.32$	48.9–49.8
Valve 3	50.1	$\pm 0.62$	49.3–51.0
Valve 4	49.7	$\pm 0.53$	49.0–50.5

tribution of particles emitted by solution-type aerosols with alcoholic cosolvents. Rapid expansion and vaporization of propellant leaves the relatively high boiling alcohol to form droplets which must evaporate to yield the small particles. Mechanical devices are used to aid the break up of droplets, but pressure is still the predominant factor in determining particle distribution of the solution-type aerosol medication.

Pressure is, to a lesser extent, important in breaking up agglomerates of micronized drug particles of suspension-type aerosols. To illustrate this dependency, determination of particle size distributions for identical micronized powders emitted from containers of 45 psig<sup>2</sup> and 80 psig were made. The data are shown in Table V. Microscopic observation of powder showed some agglomerates, but no individual particles greater than 5  $\mu$ .

TABLE V.—EFFECT OF INTERNAL PRESSURE OF ISOPROTERENOL SUSPENSION ON PARTICLE SIZE DISTRIBUTION EMITTED

Sample	Pressure, psig	Mass with Diameters		
		MMD, $\mu$	<5 $\mu$ , %	<7 $\mu$ , %
55202	80	1.9	99.6	100.0
	45	3.7	69.0	89.0
55200	80	2.5	83.1	95.4
	45	4.0	66.2	89.0

Determination of pressure is made by use of an accurate gauge fitted with a hypodermic needle. The container held in a water bath is pierced with the hypodermic needle, the vial and gauge tapped lightly, and reading is made. If a gas tight seal is not made between needle and inside of the container, the aerosol can be cooled to  $-40^\circ$ , transferred to a cold 10-cc. plastic-coated glass vial, and sealed with a multiple-dose neoprene stopper and aluminum closure. Pressure readings can then be readily made through the aluminum and rubber closure with the hypodermic needle pressure gauge.

<sup>2</sup> Pounds per square inch, gauge.

## CONCLUSIONS

Reproducibility of dosage and consequent uniformity of therapeutic effect of pressurized aerosols for inhalation can be achieved when adequate control is exercised over particle size, valve delivery, and pressure of the finished preparation. Considering that only microgram quantities of medication are delivered, tolerances for variation are comparable to other oral dosage forms. Reproducibility is much superior to oral liquid preparations dispensed by teaspoon or dropper, and compares favorably with weight variation permitted for hard gelatin capsules (16).

## REFERENCES

- (1) Goldstein, M. M., Attinger, E. W., and Hapner, I., *Ann Allergy*, 15, 626(1957)
- (2) Harris, M. C., *Postgraduate Med.*, 23, 170(1958)
- (3) Grater, W. C., and Shuey, C. B., *Southern Med J.*, 51, 1600(1958)
- (4) Freedman, T., *Postgraduate Med.*, 20, 667(1956)
- (5) Zohman, L., and Williams, M. H., Jr., *J Allergy*, 29, 72(1958)
- (6) Seltzer, A., *Med Ann Dist Columbia*, 27, 131(1958)
- (7) Sciarra, J. J., *Aerosol Age*, 1, 14(Sept 1956)
- (8) Root, M. J., *ibid.*, 1, 30(May 1956)
- (9) Barr, M., *ibid.*, 2, 19(Sept 1957)
- (10) Dauterbande, L., *Physiol Revs.*, 37, 214(1952)
- (11) Findeisen, W., *Arch ges physiol Pfluger's*, 236, 367(1933)
- (12) Abramson, A. H., *Progr in Allergy*, 2, 84(1949)
- (13) Pilcher, J. M., Mitchell, R. I., and Thomas, R. T., *Proc Chem Specialties Mfg Assoc*, 1955, 74
- (14) Tarpley, W. B., *Aerosol Age*, 2, 38(1958)
- (15) Dimmick, R. L., Hatch, M. T., Ng, J., *A M A Arch Ind Health*, 18, 23(1958)
- (16) "United States Pharmacopeia XV," Mack Publishing Co., Easton, Pa., 1955, p. 945

## Pressurized Pharmaceutical Aerosols for Inhalation Therapy II\*

### Analytical Control Methods

By JAMES G. YOUNG, IRVING PORUSH, CHARLES G. THIEL, SHELDON COHEN, and C. HOWARD STIMMEL

Analytical procedures for the control of pressurized aerosols for inhalation therapy require techniques not usually encountered in other dosage forms. Procedures are described for assay of active ingredients, amount of medication delivered per dose, and moisture content. Claims for improved stability of epinephrine and isoproterenol in this new dosage form are supported by data obtained from chemical and biological stability studies. Uniformity of dosage delivered from the aerosol valve and from the adapter was determined by chemical analyses and found to be well within the acceptable limits for other dosage forms.

THE ANALYTICAL CONTROL of pressurized aerosols for oral inhalation therapy involves techniques not usually encountered in other dosage forms. It is the purpose of this communication to describe some of these techniques and to provide data which emphasize two of the advantages of pressurized aerosols, *viz.*, improved stability of medicament and uniformity of dosage.

Data are presented on two aerosols, Medi-haler-Epi and Medi-haler-Iso, which contain epinephrine bitartrate and isoproterenol sulfate, respectively, suspended in a mixture of fluorochlorohydrocarbons with a surfactant as dispersing agent.<sup>1</sup>

\* Received August 21, 1959, from the Riker Laboratories, Inc., Northridge, Calif.

<sup>1</sup> Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

Medi-haler-Epi (Riker Laboratories, Inc.) contains 7 mg of epinephrine bitartrate per cc of suspension. Medi-haler-Iso (Riker Laboratories, Inc.) contains 2 mg of isoproterenol sulfate per cc of suspension.

### SPECIFICATIONS OF PROPELLENTS

**Identification.**—The fluorochlorohydrocarbons used as propellents are identified by infrared spectrophotometric analysis of a completely volatilized sample.

**Inorganic Fluoride.**—Products used internally must be free of inorganic fluoride, with a maximum limit of 10 p.p.m. considered acceptable. Tests for fluoride ion that could be a product of hydrolysis of the fluorochlorohydrocarbons are essential. The analysis of inorganic fluoride is carried out on an aqueous extract of propellant using the micro method of spontaneous electrolysis according to Baker and Morrison (1).

**High Boiling Residue.**—A sample of each propellant is evaporated to dryness. The high boiling residue is determined gravimetrically and must not exceed 0.05%.

### DETERMINATION OF MOISTURE

One of the factors which contributes to the stability of epinephrine and isoproterenol in this dosage

form is the exclusion of moisture. The maximum tolerance for total moisture is 250 p. p. m. for Medihaler-Iso, which contains 100 p. p. m. as water of hydration, and 150 p. p. m. for Medihaler-Epi.

The method used in this laboratory is that of Karl Fischer (2, 3). A sample of aerosol suspension is added to a measured amount of Karl Fischer (K. F.) reagent and the excess reagent is then back titrated with a standard solution of water in methanol.

Standardization of the K. F. reagent and the titration of the sample are carried out in a clear-glass plastic-coated bottle containing a magnetic stirring bar and sealed with a multiple-dose neoprene closure. To prevent interference from atmospheric moisture during standardization of the K. F. reagent, a positive pressure (ca. 5 psig)<sup>2</sup> is maintained in the titration bottle by the addition of a small amount of propellant 114. Addition of the propellant or sample to the titration bottle is accomplished by transfer from a warmed bottle to a chilled bottle using a double-ended 20-gauge hypodermic needle. A mixture of formamide:methanol (1:1, ca. 4 ml.) is used to solubilize the propellants in a 4-5-Gm. sample of aerosol suspension.

Titration is made with hypodermic syringes equipped with 24-gauge needles and graduated to 0.05 ml. or less. The end point of a titration is determined electrometrically using the titrimeter circuit described by Reed (4). The platinum wire electrodes are inserted through the neoprene seal of the titration bottle with the aid of a 20-gauge hypodermic needle.

## ASSAY PROCEDURES

**Assay of Epinephrine Bitartrate.**—The gross weight of the intact aerosol is determined accurately. Using a sharp tool, the top of the closure is punctured and a 25-gauge hypodermic needle is inserted. The gaseous propellant is allowed to escape until the internal pressure has been reduced to a point where the container can be opened safely. The closure is removed and the residual suspension poured onto a small sintered-glass filter of fine porosity. The dispersing agent is removed from the solid epinephrine bitartrate by washing the container and valve with 25 ml. of carbon tetrachloride in several small portions, adding the washings to the filter. After each transfer from container to filter, the solid residue is allowed to settle on the filter with the application of light suction. The filtrate is discarded. The container and valve are washed by the above technique using about 50 ml. of distilled water. The filtrate is collected, transferred quantitatively to a 100-ml. volumetric flask, and brought to volume with distilled water. The absorbance at 278  $m\mu$  of an appropriate dilution is compared to that of a standard solution of epinephrine bitartrate U. S. P. The empty aerosol vial and valve are dried and their combined tare weight determined. Using the spectrophotometric data and the net weight of the aerosol, the concentration of epinephrine bitartrate in the aerosol is computed.

The absorption spectrum from 400 to 210  $m\mu$  of the final dilution must conform to that of a standard solution of epinephrine bitartrate U. S. P.

The aerosol of isoproterenol sulfate is assayed by

the same procedure except that 0.01 *N* sulfuric acid is used as the solvent.

## STABILITY OF MEDICAMENT

One of the advantages of this aerosol preparation is that the active ingredients are protected from atmospheric oxygen. This is pertinent in the case of the catechol amines which are known to be susceptible to oxidative deterioration. Also, the stability of these compounds is further enhanced by the fact that they are present as completely insoluble suspensions in a nonaqueous medium.

The excellent stability of epinephrine bitartrate and isoproterenol sulfate is demonstrated by the data in Tables I and II. To assure that the spectrophotometric assays are a true measure of active catechol amine, bioassays are included.

TABLE I.—STABILITY OF EPINEPHRINE BITARTRATE

	Per Cent of Initial Concentration 12 Months		24 Months Room Temperature
	Room Temperature	45°	
Chemical <sup>a</sup>	100.5	100.4	97.5
Bioassay <sup>b</sup>	98.8	108.3	99

<sup>a</sup> Average of determinations on three aerosols.

<sup>b</sup> Method of U. S. P. XV.

TABLE II.—STABILITY OF ISOPROTERENOL SULFATE

	Per cent of Initial Concentration 12 Months		24 Months Room Temperature
	Room Temperature	45°	
Chemical <sup>a</sup>	95.5	91.5	93.5
Bioassay <sup>b</sup>	117.2	118.3	94

<sup>a</sup> Average of determinations on three aerosols.

<sup>b</sup> Depressor effect of isoproterenol was measured using modification of method for epinephrine U. S. P. XIV.

## UNIFORMITY OF DOSAGE

In a previous communication (5) data were presented showing the reproducibility of weights of individual shots taken at intervals from a single aerosol. However, since these preparations are suspensions it remained to be proved that a uniform shot-weight represents a uniform dosage of active ingredient. This has been accomplished by chemical assay of the active component delivered in each of several shots taken at regular intervals from an aerosol vial.

**Delivery of Active Ingredient from Valve.**—The aerosol was placed inverted in the bottom of a 250-ml. glass-stoppered cylindrical graduate containing exactly 200 ml. of 0.1 *N* acetic acid. Using a glass rod pressed against the bottom of the vial, the aerosol was discharged by pressing the stem of the valve against the bottom of the graduate. The rod was carefully dropped into the graduate which was quickly stoppered and inverted twenty-five times. This technique has the advantage of extracting the gas phase which appears as a cloud above the surface of the liquid when the aerosol is discharged. A 10-ml. aliquot of the acetic acid solution was assayed for catechol amine by the method of Weil-Malherbe and Bone (6) using the fluorimetric attachment of the Beckman DU spectrophotometer equipped with

<sup>2</sup> Pounds per square inch, gauge.

a primary filter, Wratten No. 50, and a secondary filter, Wratten No. 15(G). With each aerosol, every tenth shot was taken arbitrarily with a total of 21-22 shots being collected and assayed. This procedure effectively empties the aerosol. The results are shown in Table III.

TABLE III.—UNIFORMITY OF DOSAGE

Mcg. Epinephrine Bitartrate/Valve Depression	Mcg. Isoproterenol Sulfate/Valve Depression
312	104
307	104
339	97
393	97
344	101
295	99
335	98
329	96
342	97
339	91
326	96
306	93
296	97
301	95
323	94
344	97
312	97
325	93
293	98
285	100
394	95
360	
Mean 327.3	Mean 97.1
S. D. 29.3	S. D. 3.3

**Delivery of Active Ingredient from Adapter.**—Oral inhalation of a drug from an aerosol requires the use of a special adapter which is inserted into the mouth before discharging the aerosol. A certain

amount of drug is trapped by the adapter, thereby reducing the amount of drug delivered to the patient. To determine the amount of drug trapped by the adapter and the uniformity of dose delivered to the patient, the apparatus pictured in Fig. 1 was used. The aerosol with adapter was discharged into a funnel connected by Tygon tubing to a glass tube with a sintered plate immersed in a gas-washing bottle containing exactly 75 ml. distilled water. The second tube from the gas-washing bottle was connected to a vacuum line which pulled 16 L./min. air through the system as the aerosol was discharged. Thus, each dose was delivered through the adapter under conditions emulating inhalation by the patient. After each shot, the solution in the gas-washing bottle was transferred to a 200-ml. volumetric flask. The funnel, tubing, and bottle were washed with several portions of distilled water and the washings added to the volumetric flask. Sufficient glacial acetic acid was added to the flask to give 0.1 N solution when brought to volume. A 10-ml. aliquot of the final solution was assayed fluorimetrically as described previously. Every ten shots with a total of ten shots was collected and assayed. The results are shown in Table IV.

TABLE IV.—UNIFORMITY OF DOSAGE THROUGH ADAPTER

Epinephrine Bitartrate Delivered From Adapter per Valve Depression, mcg.	
250	302
280	265
246	331
250	268
243	287
Mean 272	
S. D. 28	

The average amount of drug delivered through the adapter was 77.9% of the theoretical amount calculated from volume delivered by the valve.

SUMMARY

Pressurized aerosols for the administration of drugs have received considerable acclaim because of their many advantages. However, the literature has given little attention to the means whereby these advantages become manifest, particularly in aerosols which are made to deliver small doses of potent drugs for internal use. The latter require the most exacting standards for their control and manufacture.

The data presented here and in a previous communication (5) represent an attempt to establish some standards for this dosage form.

REFERENCES

(1) Baker, B. B., and Morrison, J. D., *Anal. Chem.*, **27**, 1306(1955).  
(2) Fischer, K., *Z. Angew. Chem.*, **48**, 394(1935).  
(3) Mitchell, J., and Smith, D. M., "Chemical Analysis Series, Aquametry," Vol. V., Interscience Publishers, Inc., New York, N. Y., 1948.  
(4) Reed, F. T., *Refrig. Eng.*, **62**, No. 7, 65(1954).  
(5) Porush, I., Thiel, C. G., and Young, J. G., *THIS JOURNAL*, **49**, 70(1960).  
(6) Weil-Malherbe, H., and Bone, A. D., *Biochem. J.*, **51**, 311(1952).

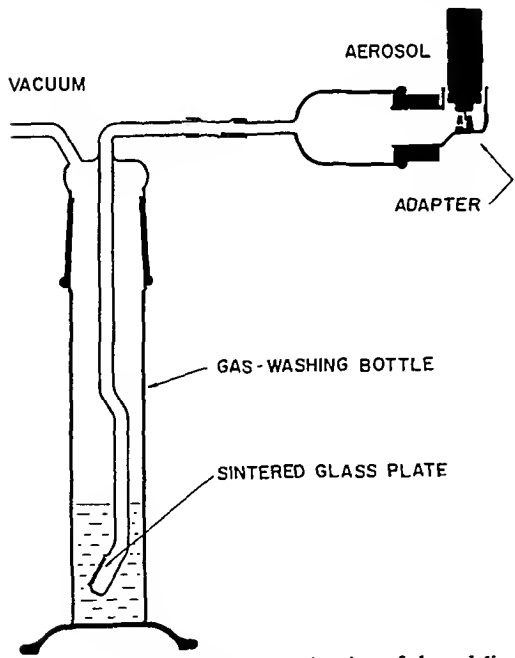


Fig. 1.—Apparatus for determination of dose delivered from adapter.

# A Comparative Study of Pharmaceutical Emulsification Equipment\*

By GARNET E. PECK, H. GEORGE DEKAY, and GILBERT S. BANKER

A study has been made of six common types of emulsification equipment normally encountered in pilot plant experimentation or small-scale manufacturing. Mineral oil and cod liver oil emulsions were prepared. The evaluation of the emulsion products involved measurements of particle size distribution, rate of creaming, and viscosity. The particle size distribution studies and the rate of creaming evaluation clearly differentiated the equipment studied. It was shown that the emulsions processed with a homogenizer usually produced samples with a smaller average particle size and a lower rate of creaming under the conditions of the study. The data of this study were subjected to statistical evaluation which indicated that both the emulsifying agent and the processing equipment used significantly affect emulsion quality. A significant interaction between equipment and emulsifying agents was also shown.

THE PREPARATION and evaluation of liquid emulsified products present unique and important problems to the pharmaceutical industry. To economically manufacture stable and pharmaceutically elegant emulsion products, the best power equipment should be selected for the required processing, and then this equipment should be used in an efficient manner.

In this investigation six common types of emulsification equipment were used to prepare various emulsion samples. The samples were observed for their (a) degree of creaming, (b) apparent viscosity, and (c) particle size distribution of the dispersed phase. The results were then statistically correlated to the equipment utilized for the various formulations studied.

## EXPERIMENTAL

**Emulsification Equipment and Standardization.**—The equipment used in this study may be divided into the three groups as stated by Johnson (1). Table I illustrates the equipment used and the conditions under which they were operated. The wall mixer was equipped with a 34.5-cm. shaft, a 7.6-cm. two blade marine-type propeller, and a 1/4-hp motor, which was mounted vertically on a movable frame. The container used with this mixer had a diameter of 20.3 cm and a height of 24.5 cm. The mixer shaft was vertically inserted 5.5 cm. from the edge of the container. For the Eppenbach Homo Mixer, which had a 5.5-cm. propeller, a container with a diameter of 16.3 cm and a height of 19.3 cm. was used. The mixing shaft was inserted vertically and placed at the center of the container during the mixing.

In this study the r. p. m. of all equipment except the Manton Gaulin homogenizer were recorded and maintained at a definite level. The measurements were taken with a Strobatae.<sup>1</sup> For the standardization of both equipment and emulsion preparation it was necessary to prepare preliminary emulsions.

TABLE I.—EQUIPMENT SPEEDS AND SETTINGS

Equipment	Speed, r. p. m.	Setting
<b>Agitators</b>		
Wall mixer <sup>a</sup>	1,700 <sup>b</sup>	
Waring Blendor <sup>c</sup>	10,000	1 <sup>d</sup>
Eppenbach Homo Mixer <sup>e</sup>	1,700 and 7,000 <sup>b</sup>	
<b>Colloid Mills</b>		
Tri-Homo disperser <sup>f,g</sup>	7,000	0.005 and 0.003 in.
Eppenbach colloid mill <sup>f,g</sup>	7,000	0.005 and 0.003 in.
<b>Homogenizer</b>		
Manton Gaulin homogenizer <sup>f,h</sup>	...	3,000 and 8,000 p. s. i.

<sup>a</sup> Conn. time N Y  
<sup>b</sup> ...  
<sup>c</sup> ...  
<sup>d</sup> Mixing time, three minutes  
<sup>e</sup> Gifford-Wood Co., New York 17, N. Y.  
<sup>f</sup> Emulsions processed through equipment once.  
<sup>g</sup> Tri-Homo Corp., Salem, Mass.  
<sup>h</sup> Manton-Gaulin Manufacturing Co., Inc., Everett, Mass.

From these emulsions the equipment speeds and settings were established for this study as shown in Table I. The settings in Table I for the Manton Gaulin homogenizer were for the first valve. For the second stage valve the setting used was 500 p. s. i. in all cases. For all the processing equipment the samples were collected while the equipment was at top speed or at a constant pressure.

**Particle Size Evaluation.**—A direct microscopic measurement of the globule diameter of the dispersed phase was performed using a stand microscope equipped with a 15X eyepiece and a 43X objective. The eyepiece contained a micrometer with a 5-mm. horizontal scale divided into 100 divisions. In order to examine the emulsions microscopically it was necessary to dilute them with some liquid which would not affect the dispersed phase, but would decrease Brownian movement. A 50% aqueous solution of propylene glycol was used for this purpose, based on the findings of Levius and Drommond (2). The samples were diluted in a standard spot plate from ten to twentyfold, the finer emulsions requiring the larger dilution. One drop of the diluted emulsion was then placed on a microscope slide, a cover glass was placed over the drop, and the slide allowed to stand at least fifteen minutes on the microscope stage before viewing.

\* Received August 21, 1959, from Purdue University, School of Pharmacy, Lafayette, Ind.  
 Presented to the Scientific Section, A. P. A., Cincinnati Meeting, August 1959.  
<sup>1</sup> General Radio Corp., Cambridge, Mass.



To determine the sample size (number of particles to be measured) in order to obtain a reasonably accurate estimation of population parameters, the method of Harris, Horvitz, and Moon (3) was applied to preliminary data representing the range of emulsion products anticipated for the study. With the test a maximum confidence interval of one limit, a 99% confidence coefficient, and a 95% probability were employed, with 99 degrees of freedom. A sample size of 400 particles or globules was indicated as adequate under these test conditions, and 400 particle size measurements consequently were made to obtain each emulsion mean particle size.

**Viscosity Measurements.**—In order to observe any change in apparent viscosity between samples or after aging, a Brookfield Synchroelectric, Model RVF viscometer<sup>2</sup> was used with the No. 1 spindle at a speed of 10 r p m. As indicated by Tober and Autian, the viscometer was operated for ten minutes in the sample before a reading was taken (4). All measurements were made at 25°.

**Formulation.**—The first emulsion prepared was the official liquid petrolatum emulsion N F X (5). However, it was necessary to reduce the quantity of acacia from the official 12.5% to 5.0% as suggested by Levius and Drommond (2). The emulsions prepared with power equipment were much too thick when the higher percentage of acacia was used. For the second official emulsion (cod liver oil emulsion N F X) the acacia was also reduced from 12.5 to 5.0% (6). The third emulsion prepared was an emulsion of liquid petrolatum using a mixture of 1.5% Tween 80<sup>3</sup> and 3.5% Arlacel 80<sup>3</sup> as the emulsifying agent. The fourth emulsion was a liquid petrolatum emulsion using the U S P method for emulsification (7).

**Manufacturing Directions for Emulsions.**—With the exception of the U S P gelatin method the emulsions were mixed as follows: (a) The oil and the emulsifying agent were mixed for one minute at 1,000 r p m. (b) The proper volume of water was then added all at once. The primary emulsions were formed at 1,700 r p m over a three minute period (7,000 r p m used for the Eppenbach Homo Mixer). (c) The remaining ingredients were added, water added to volume desired, and then mixed for five minutes at 1,700 r p m (7,000 r p m used for the Eppenbach Homo Mixer).

For the liquid petrolatum emulsions prepared by the gelatin method the prescribed U S P procedure was followed. The final mixing of these emulsions was accomplished at 1,700 r p m for the wall mixer and 7,000 r p m for the Eppenbach Homo Mixer.

**Machine Processing Schedule.**—Table I gives the setting and speeds used for the various samples. All samples that were processed with the refining equipment were passed through the equipment once, as this is the ideal method of equipment utilization, permits more absolute equipment comparison, and establishes a standard procedure which may be easily maintained.

**Tube Sedimentation Method.**—A variety of methods for the study of sedimentation or creaming of emulsified products was found in the literature. In this study Pyrex 20 mm external diameter tubing was cut into 18 inch lengths. To close the tubes, rubber stoppers, size No. 3 were used. Each tube was

calibrated to 100 ml using a 100 ml volumetric flask and distilled water. These tubes were stored at room temperature in special tube racks.

**Photomicrographs.**—To supplement the particle size measurements photomicrographs were taken using a standard microscope and an Eastman Kodak attachment.<sup>4</sup> The magnification of the microscope was 430X. The exposure times most frequently employed were three and five seconds.

## RESULTS AND DISCUSSION

In order to present the results of this study more efficiently, it is necessary to use a coding system for the oils, emulsifying agents, and equipment utilized in this work. Table II explains the various symbols used.

**Temperature of Emulsions After Processing.**—During the preparation of the emulsions the processing equipment was standardized for use as described under *Experimental*. As a portion of the equipment evaluation, the temperature rise produced during processing was considered. The temperature of each emulsion was taken immediately following its preparation. Two pieces of equipment were equipped with cooling jackets: the Tri-Homo disperser and the Eppenbach colloid mill. The room temperature was 25°. Table III illustrates the temperatures of the emulsions after processing.

From Table III it can be seen that the Eppenbach Homo Mixer at 7,000 r p m produced the greatest temperature rise in all of the emulsions studied. No apparent changes due to the temperature differences were observed.

**Apparent Viscosity Studies.**—All emulsion viscosity measurements were performed twenty-four hours after emulsion preparation or processing. The viscosity measurements were repeated after one

TABLE II—CODING OF SAMPLES AND EQUIPMENT

Material	Coding	Definition
Oil	LP	Liquid petrolatum
Oil	CD	Cod liver oil
Emulsifier	A	Acacia
Emulsifier	TA	Tween 80-Arlacel 80
Emulsifier	G	Gelatin
Equipment	WM	Wall mixer
Equipment	WB	Waring Blendor
Equipment	T5	Tri-Homo disperser, 0.005 in
Equipment	T3	Tri-Homo disperser, 0.003 in
Equipment	E5	Eppenbach colloid mill, 0.005 in
Equipment	E3	Eppenbach colloid mill, 0.003 in
Equipment	MG3	Manton Gaulin homogenizer, 3,000 p s i
Equipment	MG8	Manton Gaulin homogenizer, 8,000 p s i
Equipment	HM 1,700	Eppenbach Homo Mixer, 1,700 r p m
Equipment	HM 7,000	Eppenbach Homo Mixer, 7,000 r p m

<sup>2</sup> Brookfield Engineering Laboratories, Stoughton, Mass.  
<sup>3</sup> Atlas Powder Co., Wilmington 99, Del.

<sup>4</sup> Eastman Kodak Co., Rochester, N. Y.

TABLE III.—EMULSION TEMPERATURES AFTER PROCESSING, ° C.

Emulsions					Equipment		MG3	MG8	HM 1,700	HM 7,000
	WM	WB	T5	T3	E5	E3				
LP-TA	28	28	25	25	25	25	32	39	29 5	42
LP-A	27	30	25	25	25	25	29	38	29 8	41
LP-G	34	32	24	24	23	23	31	37		38
CD-A	28	30	25	25	25	25	31	40		48

month of aging at room temperature. Viscosity measurements of the emulsions prepared with acacia or the Tween-Arlacel combination were also made after two months of aging. In general, there were no appreciable changes in apparent viscosity after aging. The emulsions from the LP-G series showed little variation in apparent viscosity as a result of the equipment used. When emulsions of liquid petroleum and cod liver oil were prepared with acacia, the Eppenbach Homo Mixer produced the least viscous product.

**Rate of Creaming Studies.**—To obtain a rate of creaming for the emulsion samples, the samples were observed during shelf aging in the tubes at room temperature and the degree of separation recorded by the measurement of the aqueous layer. This measurement was then plotted against time as illustrated by Fig. 1. From graphs such as this, the sedimentation or creaming velocity was calculated according to Stokes' law. The results of the sedimentation velocity study are given in Table IV. These values have been evaluated with caution since the slope of some of the curves was determined from a line with only two points. It can be said that the Manton Gaulin homogenizer at 8,000 p. s. i. produced emulsions which had the slowest rate of creaming in all cases and thus produced the best appearing emulsions on aging. In the case of the cod liver oil emulsions the degree of creaming was greatly reduced in all samples. It was not possible to determine the sedimentation velocity for the liquid-gelatin series due to a delayed separation of the emulsion samples.

**Particle Size Measurements and Their Statistical Evaluation.**—Following the measurement of 400 globules of each emulsion studied, the average par-

TABLE IV.—SEDIMENTATION VELOCITIES OF THE EMULSIONS IN CM/DAY

Equipment	LP-A Series	LP-TA Series	CD A Series
	V	V	V
WM	4 10	5 00	1 50
WB	0 12	0 80	0 01
T5	0 40	2 10	0 30
T3	0 23	1 96	0 14
E5	0 24	2 10	0 30
E3	0 21	1 97	0 11
MG3	0 17	0 76	0 01
MG8	0 12	0 00	0 01
HM 1,700	2 37	1 18	
HM 7,000	0 17	2 90	0 33

$$\text{Example, LP - A - WM: } V = \frac{h_2 - h_1}{t_2 - t_1} = \frac{12.3 - 0}{3.0 - 0} = 4.10$$

ticle size and the standard deviation of the samples were determined. Tables V and VI are the summation of all these determinations. It can be seen that in most cases those samples prepared initially with the wall mixer had the largest average apparent particle size. Differences in the average particle size between the readings were shown not to be statistically significant.

An analysis of variance was performed to assist in the interpretation of the particle size distribution studies. A three-factor analysis of variance was conducted, with  $i = 1-3$  for the three emulsifying agents,  $j = 1-2$  for the two aging periods (initially and one month later), and  $k = 1-8$  for the equipment and settings utilized, according to the  $i, j, k$  notation and design of Ostle (8). Experimental replication indicated good reproducibility of the results. It should be noted that the results from the wall mixer and the Eppenbach Homo Mixer at 1,700 r p m were not included. These samples had rather large average particle sizes and thus were obviously significantly different from all other samples. Table VII illustrates the results of the analysis of variance. From the table it can be seen that at the 5% level, significant differences are indicated between emulsifying agents, between equipment, and with the equipment-emulsifying agent interaction. In other words, the analysis of variance indicates that there are significant differences in average emulsion particle size depending on which emulsifying agent is used, which piece of processing equipment is used, and which piece of processing equipment is used with which emulsifier. The differences in average particle size with aging were not statistically significant, nor were the reading interactions significant.

To determine individual differences in average emulsion particle size of various pieces of processing equipment with each formulation studied, the Newman-Kuels test was employed (9). This test is valuable for making multiple nonindependent signifi-

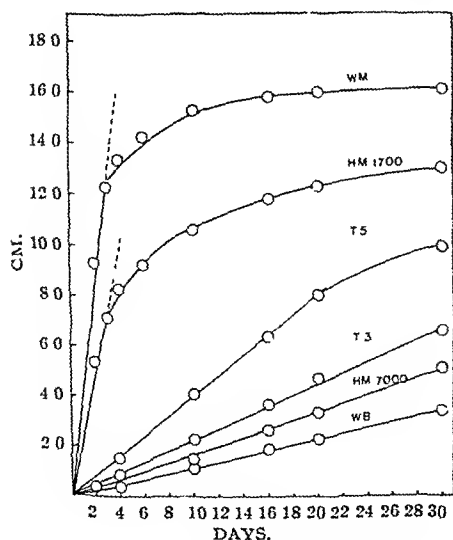


Fig. 1.—Rate of creaming, LP-A series.

TABLE V.—PARTICLE SIZE MEASUREMENTS FOR THE LP-A AND LP-TA SERIES

Sample	1st Reading <sup>a</sup>		2nd Reading <sup>b</sup>		3rd Reading <sup>c</sup>	
	$\bar{X}^d$	S <sup>e</sup>	$\bar{X}$	S	$\bar{X}$	S
LP-A-WM <sub>1</sub>	13.452	15.026	9.516	18.847	16.643	20.533
LP-A-WB	5.294	2.560	5.312	1.369	5.447	1.672
LP-A-T5	5.191	7.341	5.745	3.138	4.214	2.631
LP-A-T3	4.018	2.271	3.941	2.391	3.734	2.156
LP-A-E5	5.857	7.080	7.507	6.854	5.921	3.898
LP-A-E3	5.100	1.897	6.361	7.277	5.426	2.802
LP-A-MG3	3.562	1.990	2.801	1.940	4.058	4.131
LP-A-MG8	4.453	1.761	4.875	3.498	4.904	5.050
LP-A-HM 1,700	13.485	12.515	7.036	7.438	4.342	7.197
LP-A-HM 7,000	3.815	1.390	3.799	3.063	3.367	0.976
LP-TA-WM	4.190	6.816	2.223	2.601	1.056	1.984
LP-TA-WB	2.600	5.366	2.133	0.616	1.700	0.672
LP-TA-T5	5.070	1.819	2.785	2.458	1.843	2.454
LP-TA-T3	4.410	3.003	4.103	3.354	1.593	2.118
LP-TA-E5	3.900	2.698	2.855	3.724	2.033	1.678
LP-TA-E3	4.300	3.719	1.748	2.032	1.892	1.885
LP-TA-MG3	1.400	0.399	1.320	0.478	1.086	0.324
LP-TA-MG8	1.030	0.262	0.930	0.442	0.918	0.175
LP-TA-HM 1,700	3.880	3.139	2.228	2.600	1.520	3.233
LP-TA-HM 7,000	2.091	2.223	1.815	0.488	1.457	0.531

<sup>a</sup> Initial measurement. <sup>b</sup> After one month. <sup>c</sup> After two months. <sup>d</sup> In microns. <sup>e</sup> Standard deviation. <sup>f</sup> See Table II for code.

TABLE VI.—PARTICLE SIZE MEASUREMENTS FOR THE LP-G AND THE CD-A SERIES

Sample	1st Reading <sup>a</sup>		2nd Reading <sup>b</sup>	
	$\bar{X}^c$	S <sup>d</sup>	$\bar{X}$	S
LP-G-WM <sup>e</sup>	19.148	18.763	19.288	17.622
LP-G-WB	2.823	3.173	3.782	3.503
LP-G-T5	5.881	6.582	6.820	11.554
LP-G-T3	7.090	12.314	6.585	11.133
LP-G-E5	4.245	6.654	6.149	7.998
LP-G-E3	6.058	7.265	3.542	3.771
LP-G-MG3	1.384	1.004	1.303	0.965
LP-G-MG8	1.426	0.289	1.122	0.274
LP-G-HM 7,000	3.326	2.843	3.413	2.502
CD-A-WM	5.864	7.597	8.596	8.441
CD-A-WB	1.769	0.748	1.568	0.846
CD-A-T5	4.489	2.503	5.301	4.169
CD-A-T3	3.970	1.399	4.015	2.762
CD-A-E5	2.823	1.590	3.469	1.658
CD-A-E3	2.868	2.064	4.210	1.631
CD-A-MG3	1.841	0.322	1.915	0.401
CD-A-MG8	2.296	0.028	2.474	0.288
CD-A-HM 7,000	2.159	0.989	2.075	0.801

<sup>a</sup> Initial measurement. <sup>b</sup> After one month. <sup>c</sup> In microns. <sup>d</sup> Standard deviation. <sup>e</sup> See Table II for code.

cance tests on differences among individual means in an analysis of variance. In the case of the liquid petrolatum-acacia emulsions after the first reading the following was shown: (a) No significant difference between the Waring Blendor and the Tri-Homo disperser at 0.005-inch samples was observed. (b) No significant difference between the Tri-Homo disperser at 0.005-inch and the Eppenbach colloid mill at 0.003 inch-samples was observed.

For the remainder of the samples in the series, all particle size differences between the equipment utilized were significant.

**Photomicrographs.**—Photomicrographs are presented to illustrate the particle distribution of the emulsions. In all the samples the emulsions were diluted with the same quantity of the 50% aqueous propylene glycol solution. Figure 2 is the photograph of the wall mixer sample of a liquid petro-



Fig. 2.—Liquid petrolatum—Twcwn, Arlcel emulsion: wall mixer.

tum-Twccn-Arlcel emulsion. Figure 3 is the photograph of this formulation processed with the Manton Gaulin homogenizer at 8,000 p. s. i. From these two figures it can be seen that there is a definite increase in the number of globules per unit area as the size of the globules is reduced.

TABLE VII.—ANALYSIS OF VARIANCE

Source	Sum of Squares	Degrees of Freedom	Mean Square	Calculated <i>F</i>	<i>F</i> <sub>0.05</sub> <sup>a</sup>	Significant
<i>i</i> (Agents) <sup>b</sup>	39.5764	2	19.7882	34.7222	3.74	Yes
<i>j</i> (Readings) <sup>c</sup>	0.2670	1	0.2670	0.4685	4.60	No
<i>k</i> (Equipment)	71.7397	7	10.2485	17.9830	2.77	Yes
<i>i</i> × <i>j</i>	3.4820	2	1.7410	3.0549	3.74	No
<i>i</i> × <i>k</i>	28.8507	14	2.0608	3.6160	2.48	Yes
<i>j</i> × <i>k</i>	3.6306	7	0.5187	0.9102	2.77	No
<i>i</i> × <i>j</i> × <i>k</i> (error)	7.9788	14	0.5699	...	...	..
Total		47	...	...	...	..

<sup>a</sup> *F*<sub>0.05</sub> = 5% Points from the *F* distribution (9)    <sup>b</sup> Emulsifying agents.    <sup>c</sup> The effect of aging

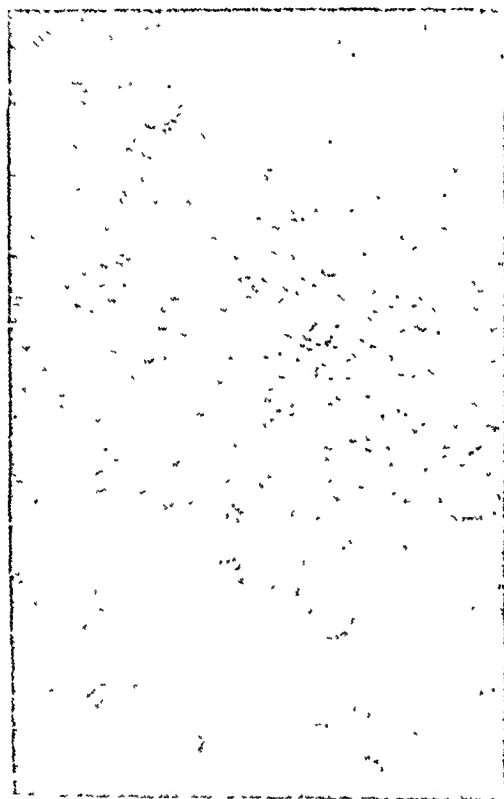


Fig. 3.—Liquid petrolatum—Tween, Arlacel emulsion: Manton Gaulin homogenizer, 8,000 p. s. i.

### CONCLUSIONS

1. When utilizing certain pieces of equipment to process several emulsion formulations, an appreciable emulsion temperature increase occurred. The Eppenbach Homo Mixer produced the greatest temperature increase, 20°.

2. Emulsions prepared with acacia were more viscous than those prepared with either gelatin or a Tween-Arlacel combination. Some differences in apparent viscosity were observed when the various pieces of equipment were utilized. Aging of the emulsion samples at room temperature did not produce appreciable changes in apparent viscosity.

3. When using a simple sedimentation or

creaming test conducted at room temperature, it was shown that the Manton Gaulin homogenizer at 8,000 p. s. i. produced the least amount of creaming in the emulsions. The rate of creaming was calculated according to Stokes' law.

4. The Manton Gaulin homogenizer was the most effective of all the equipment studied, using a decrease in particle size of the dispersed phase as the criterion for efficient emulsification processing. The homogenizer produced samples with the smallest average particle size for all four formulations studied.

5. An analysis of variance of the mean particle size data indicated that significant differences existed between (a) the three emulsifying agents, (b) the six pieces of processing equipment, and (c) the interaction between the emulsifying agents and equipment.

6. With the formulations used in this study, one pass through the homogenizer (pressure type) produced a much greater particle size reduction than any other piece of equipment used.

7. A refined oil such as cod liver oil emulsified with acacia is definitely easier to emulsify than liquid petrolatum with the same emulsifying agent, as shown by the smaller average particle size of the wall mixer samples and by the general lower rate of creaming.

In the preparation of emulsions, the emulsifying agents and the equipment to be utilized should be carefully considered in any formulation studies to obtain maximum particle size reduction.

### REFERENCES

- (1) Johnson, R. I., "Emulsion Technology," Chemical Publishing Co., Inc., Brooklyn, N. Y., 1946, pp. 89-90.
- (2) Levius, H. P., and Drommond, F. G., *J. Pharm. and Pharmacol.*, **5**, 743 (1953).
- (3) Harris, M., Horvitz, D. G., and Moon, A. M., *J. Am. Stat. Assoc.*, **43**, 391 (1948).
- (4) Tober, T. W., and Autian, J., *J. Am. Pharm. Assoc., Pract. Pharm. Ed.*, **19**, 422 (1958).
- (5) "The National Formulary," 10th ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 431.
- (6) *Ibid.*, p. 170.
- (7) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1955, p. 809.
- (8) Ostle, B., "Statistics in Research; Basic Concepts and Techniques for Research Workers," The Iowa State College Press, Ames, Iowa, 1954, p. 258.
- (9) Duncan, D. B., *Biometrics*, **11**, 3 (1955).

# The Synthesis and Pharmacology of N-(Substituted Aminoacyl)-chlorotoluidines II\*

By ELIAS EPSTEIN and NICHOLAS MALATESTINIC

A number of N-(substituted aminoacyl)-chlorotoluidines were prepared and tested for local anesthetic potency, toxicity, and irritation on laboratory animals. Several of these compounds, as well as a few previously described, were tried clinically for local anesthesia in dental procedures.

THE PROPERTY of inducing local anesthesia is widely spread among the various nitrogen-containing organic compounds. A great number of amino derivatives of esters, amides, alcohols, and hydrocarbons have been prepared and their local anesthetic effect noted in the literature.

In our continuing search for better local anesthetics, we had described in our previous paper (1) several N-(substituted aminoacyl)-chloroanilines and toluidines, some of which showed sufficient promise to warrant further investigation of this chemical structure. Additional compounds of this type, as well as a few N-ethyl chlorotoluidines, were prepared. After having been tested for anesthetic potency, toxicity, and for irritation on laboratory animals, several compounds from this as well as from the previously prepared group were tried clinically for local anesthesia in dental procedures.

The general method of preparation consisted of treating a chloroacyl chloride with a chlorotoluidine in a manner described by Jacobs and Heidelberger (2), with some modifications. The chlorotoluidide was treated with excess amine to form the anesthetic base which was isolated and purified by distillation under high vacuum of between 10 and 40 $\mu$ . The anesthetics were isolated as their hydrochloride salts. Table I lists the boiling point of the bases and the melting point and analysis of the chlorotoluidide hydrochlorides.

## EXPERIMENTAL

**N-Ethyl-3-chloro-4-methylaniline.**—A mixture of 169 Gm. (1.2 mole) of 3-chloro-4-methylaniline, 166 Gm. of diethyl sulfate, and 960 ml. of 2 *N* sodium hydroxide was heated at reflux temperature, with stirring, for one hour. After the mixture was saturated with sodium chloride, the upper oil layer was separated and dissolved in 500 ml. of 3 *N* hydrochloric acid. A solution of 83 Gm. of sodium nitrite in

500 ml. of water was added slowly, with stirring. The reaction temperature was kept under 10°. The precipitate which formed was filtered and washed consecutively with water, 3 *N* hydrochloric acid, and then water. The dried precipitate was dissolved in 200 ml. of ethyl alcohol. The solution was saturated with hydrogen chloride and heated at reflux temperature for two hours. On evaporation of the alcohol, 75 Gm. (37%) of crude N-ethyl-3-chloro-4-methylaniline hydrochloride was obtained as a white solid.

**$\alpha$ -Chloro-3-chloro-4-methyl-N-ethyl-acetanilide.**—To 75 Gm. (0.3 mole) of crude N-ethyl-3-chloro-4-methylaniline hydrochloride in 250 ml. of glacial acetic acid was added, with stirring, 40 Gm. (0.36 mole) of chloroacetyl chloride. The mixture was stirred for ten minutes and 200 Gm. of sodium acetate, dissolved in 500 ml. of water, was added all at once, with strong stirring. The slurry, after stirring for thirty minutes, was filtered and washed with water. The precipitate was dissolved in ether and the solution was extracted with dilute ammonium hydroxide and then water. After having been dried over anhydrous sodium sulfate, the ether solution was distilled. The fraction at 125–135° at 70  $\mu$  yielded 60 Gm. (72%) of the anilide as a light yellow oil.

**Illustrative General Procedure for Table I Compounds.**—A mixture of 15 Gm. (0.06 mole) of  $\alpha$ -chloro-3-chloro-4-methyl-N-ethyl-acetanilide and 22 Gm. (0.3 mole) of isobutylamine was heated at reflux temperature for twelve hours. The excess isobutylamine was evaporated on a steam bath and the residue treated with excess 5% hydrochloric acid. The mixture was extracted three times with ether. The water layer was made alkaline with excess ammonium hydroxide and extracted with ether. The ether solution was distilled and the fraction boiling at 110–120° at 20  $\mu$  yielded 9.8 Gm. (56%) of a clear liquid. An ether solution of the base was neutralized with anhydrous hydrochloric acid and the precipitate recrystallized from ethyl acetate to yield  $\alpha$ -isobutylamino-3-chloro-4-methyl-N-ethyl-acetanilide hydrochloride as white crystals, m. p. 164–165°.

## PHARMACOLOGY


A pharmacological screening for use as local anesthetics of these compounds as well as those previously published (1) was conducted and several compounds were selected for clinical testing.

The topical anesthetic potency was determined by application to the rabbit cornea of successively lower concentrations of each compound and comparing the length of anesthesia with that given in the literature for cocaine hydrochloride (3). This method, originated by Koller and described by Hirschfelder and Bieter (4) was modified as described in a previous paper (5).

The conductive anesthetic potency was determined by blocking the sciatic nerve of the guinea pig

\* Received July 6, 1959, from the Research Laboratories of Novocol Chemical Mfg. Co., Inc., Brooklyn 7, N. Y.  
The authors are indebted to Richard Stribas for assistance in the analyses and to Michael Fisher for assistance in the pharmacological testing of these compounds.

TABLE I.—N-(SUBSTITUTED AMINOACYL)-CHLOROTOLUIDINES RNHCO(CH<sub>2</sub>)<sub>n</sub>R'

R'	$\frac{B}{C} \frac{p}{\mu}$ Base	n	$\frac{M}{C} \frac{p}{HCl}$	Formula	Ionic Cl, %		Molecular Wt	
			(1) R = 2-Methyl-3-chlorophenyl		Calcd	Found	Calcd	Found
NHCH <sub>3</sub>	120/20	1	Decompn 233	C <sub>10</sub> H <sub>14</sub> ON <sub>2</sub> Cl <sub>2</sub>	14 24	14 30	249	246
NHC <sub>2</sub> H <sub>5</sub>	140/20	1	Decompn. 273	C <sub>12</sub> H <sub>18</sub> ON <sub>2</sub> Cl <sub>2</sub>	12 78	12 95	277	278
NHC <sub>3</sub> H <sub>7</sub>	161/40	2	204-206	C <sub>12</sub> H <sub>18</sub> ON <sub>2</sub> Cl <sub>2</sub>	12 78	12 58	277	281
NHC <sub>4</sub> H <sub>9</sub>	149/30	1	Decompn 235	C <sub>12</sub> H <sub>20</sub> ON <sub>2</sub> Cl <sub>2</sub>	12 18	12 32	291	289
NH(CH <sub>2</sub> ) <sub>3</sub> OCH <sub>3</sub>		1	216-218	C <sub>13</sub> H <sub>20</sub> O <sub>2</sub> N <sub>2</sub> Cl <sub>2</sub>	11 51	11 78	307	285
Piperidino	143/30	1	227-229	C <sub>14</sub> H <sub>20</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 69	11 86	303	288
NHC <sub>4</sub> H <sub>9</sub> (iso)	168/20	2	229-230	C <sub>14</sub> H <sub>22</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 62	11 68	305	309
N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	155/20	2	135-137	C <sub>14</sub> H <sub>22</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 62	11 48	305	296
NH(CH <sub>2</sub> ) <sub>3</sub> OCH <sub>3</sub>	205/20	2	123-126	C <sub>14</sub> H <sub>22</sub> O <sub>2</sub> N <sub>2</sub> Cl <sub>2</sub>	11 04	10 97	321	316
N' Methyl piperazino		1	Decompn 235	C <sub>14</sub> H <sub>22</sub> ON <sub>2</sub> Cl <sub>3</sub>	19 99 <sup>a</sup>	20 38	355 <sup>a</sup>	349
N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	130/10	1	162-164	C <sub>16</sub> H <sub>24</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 10	11 08	319	319
N(C <sub>4</sub> H <sub>9</sub> ) <sub>2</sub> (iso)	145/20	1	165-166	C <sub>17</sub> H <sub>28</sub> ON <sub>2</sub> Cl <sub>2</sub>	10 19	10 34	348	347
			(2) R = 4-Methyl-3-chlorophenyl					
NHC <sub>2</sub> H <sub>5</sub>		1	246-248	C <sub>11</sub> H <sub>16</sub> ON <sub>2</sub> Cl <sub>2</sub>	13 50	13 26	263	266
NHC <sub>3</sub> H <sub>7</sub>		1	263-265	C <sub>13</sub> H <sub>20</sub> ON <sub>2</sub> Cl <sub>2</sub>	12 18	11 86	291	290
NHC <sub>4</sub> H <sub>9</sub> (tert)		1	Decompn 281	C <sub>13</sub> H <sub>20</sub> ON <sub>2</sub> Cl <sub>2</sub>	12 18	12 28	291	291
NHC <sub>4</sub> H <sub>9</sub>		2	216-217	C <sub>14</sub> H <sub>22</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 62	11 61	305	305
N(CH <sub>2</sub> ) <sub>3</sub> C <sub>4</sub> H <sub>9</sub>		2	175-176	C <sub>15</sub> H <sub>24</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 10	11 23	319	316
			(3) C <sub>2</sub> H <sub>5</sub> NC(=O)CH <sub>2</sub> R'					
								
NHC <sub>2</sub> H <sub>5</sub>	105/40		177-178	C <sub>13</sub> H <sub>20</sub> ON <sub>2</sub> Cl <sub>2</sub>	12 18	12 42	291	291
NHC <sub>4</sub> H <sub>9</sub> (iso)	110/20		164-165	C <sub>15</sub> H <sub>24</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 10	11 12	319	319
N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	102/10		145-146	C <sub>15</sub> H <sub>24</sub> ON <sub>2</sub> Cl <sub>2</sub>	11 10	11 20	319	319

<sup>a</sup> Dihydrochloride

TABLE II.—PHARMACOLOGY OF N-(SUBSTITUTED AMINOACYL)-ANILIDES

$$\text{R}-\text{C}_6\text{H}_4-\text{NHC}(\text{CH}_2)_n\text{R}'$$

R	R'	n	—Relative Potency—			—Rel Toxicity <sup>c</sup> —	—Irritation <sup>d</sup> —		Clinical Evaluation <sup>e</sup>	
			Topical <sup>a</sup>	Con- duc- tive <sup>b</sup>	In filtra- tion <sup>b</sup>	i p s c	Eye	Wheal		
3-Chloro	NHC <sub>2</sub> H <sub>5</sub>	1	C	B	B	0 7	0 8	0	0	
3-Chloro	N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	1	C	B	A	0 7	0 5	0	0	C
3-Chloro	N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	2	C	A	B	0 6	0 8	0	0	
3-Chloro	NH(CH <sub>2</sub> ) <sub>3</sub> OCH <sub>3</sub>	1	C	B	B	0 8	1 1	0	0	B
2-Methyl-3-chloro	NHC <sub>2</sub> H <sub>5</sub>	1	C	B	A	0 5	0 4	+	0	B
2-Methyl-3-chloro	N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	1	C	B	B	0 8	0 5	0	0	C
2-Methyl-3-chloro	N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	2	A	C	A	1 7	2 0	0	0	
2-Methyl-3-chloro	NHC <sub>4</sub> H <sub>9</sub> (iso)	1	C	B	A	0 5	0 6	0	0	
2-Methyl-3-chloro	NHC <sub>4</sub> H <sub>9</sub> (iso)	2	B	C	A	1 3	2 0	+	+	
2-Methyl-3-chloro	Piperidino	1	B	B	B	1 0	0 9	0	0	
2-Methyl-4-chloro	N(C <sub>2</sub> H <sub>5</sub> ) <sub>2</sub>	2	C	B	B	0 4	0 5	0	0	C
2-Methyl-5-chloro	NH(CH <sub>2</sub> ) <sub>3</sub> OCH <sub>3</sub>	1	B	A	B	1 7	1 3	0	0	B
2-Methyl-6-chloro	NHC <sub>4</sub> H <sub>9</sub> (tert)	1	A	A	A	1 7	2 0	0	0	
4-Methyl-3-chloro	NHC <sub>2</sub> H <sub>5</sub>	1	B	B	A	0 5	0 5	0	0	B
4-Methyl-3-chloro	NHC <sub>3</sub> H <sub>7</sub>	2	B	A	A	0 6	0 5	0	0	B
4-Methyl-3-chloro	NHC <sub>4</sub> H <sub>9</sub> (iso)	1	B	A	A	0 3	0 3	+	0	

<sup>a</sup> As compared with cocaine hydrochloride, A—more potent, B—approx equal, C—less potent<sup>b</sup> As compared with procaine hydrochloride, A—more potent, B—approx equal, C—less potent<sup>c</sup> As compared with procaine hydrochloride, the value for the LD<sub>50</sub> s c, 750 mg/Kg, i p, 250 mg/Kg<sup>d</sup> Little or no irritation, 0, moderate irritation, +, severe irritation, ++<sup>e</sup> As compared with procaine hydrochloride, A—more potent, B—approx equal, C—less potent

with successively lower concentrations of each compound and comparing the depth and length of anesthesia to that obtained with procaine hydrochloride. Although this method originally introduced by Shackell (6) approximates conditions found in clinical practice, it has been found in some instances to give an erroneous value for the anesthetic potency in humans (5).

The infiltration anesthetic potency was obtained by noting the anesthetic effect of an intradermal injection on the guinea-pig back under controlled conditions (7) and comparing the lowest concentration that will produce anesthesia under these conditions as compared with procaine hydrochloride.

The subcutaneous and intraperitoneal toxicities were determined on white mice and the upper limits of the intravenous toxicities were obtained using rabbits. Each compound was checked for irritation, both on application to the rabbit eye and on intradermal injection in the rabbit skin, by the trypan-blue test of Weatherby (8).

The concentrations of the compounds prepared for the clinic varied from 0.5 to 2% with epinephrine 1:50,000. The upper limit used was determined by the toxicity. No clinical solution more toxic than 2% procaine hydrochloride was used.

The pharmacological results of sixteen of the more promising compounds out of a total of seventy-two, together with a preliminary clinical evaluation of nine of these are listed in Table II. The results should be considered as semiquantitative since, because of the large number of compounds screened, a minimum number of animals per compound was used. An average of four, six, and eight tests were used to obtain the relative topical, conductive, and infiltration potency of each compound. An average of twenty mice per compound were used to determine the subcutaneous and intraperitoneal toxicity. The preliminary clinical evaluation was based on an average of twenty injections in the various concen-

trations for each compound, with both block and infiltration type injections being used, in a variety of dental procedures.

## DISCUSSION AND SUMMARY

Twenty additional N-(substituted aminoacyl) chlorotoluidines were synthesized and they, together with the fifty previously prepared compounds of this type, were tested pharmacologically for use as local anesthetics. Of the nine tested clinically, six proved to be approximately equal in potency to procaine hydrochloride in dental procedures. Several compounds were precluded from clinical testing because of their low solubility in isotonic solution.

Although it was difficult to correlate molecular structure with physiological activity, it was noted that, in general, those compounds more potent topically were also more toxic and that the substitution of the ethyl group for hydrogen on the aromatic nitrogen atom resulted in more irritating and less efficient local anesthetics.

## REFERENCES

- (1) Epstein, E., and Kaminsky, D., *J. Am. Chem. Soc.*, **80**, 1892(1958).
- (2) Jacobs, W. A., and Heidelberger, M., *J. Biol. Chem.*, **21**, 139(1915).
- (3) Co Tui, Preiss, A., Nevin, M. I., *Anesthesio & Analgesia*, **22**, 301(1943).
- (4) Hirschfelder, A. D., and Bieter, R. N., *Physiol. Revs.*, **12**, 190(1932).
- (5) Epstein, E., Meyer, M., and Ginsberg, H., *Anesthesio & Analgesia*, **34**, 171(1954).
- (6) Shackell, L. F., *ibid.*, **14**, 120(1935).
- (7) Epstein, E., and Kaminsky, D., *THIS JOURNAL*, **47**, 347(1958).
- (8) Weatherby, J. H., *J. Lab. Clin. Med.*, **25**, 1199(1940).

# Preparation of Compressed Tablet Granulations by the Air-Suspension Technique II\*

By DALE E. WURSTER†

A new and rapid method for preparing compressed tablet granulations by the air-suspension technique is discussed. Experimental data dealing with material losses and variations in the drug and water content of the prepared granulations are presented.

MUCH INVESTIGATIONAL EFFORT has been expended in attempts to find means for preparing compressed tablet granulations which are devoid of the inherent disadvantages of the

conventional methods. The wet method, although probably the most widely employed, has the main disadvantage of exposing the drugs to water or other solvents and usually requires a long drying time. Also, it is composed of several individual operations including blending, granulating, and grinding which necessitates a large amount of material handling. The precompression method usually eliminates the need for wetting the drugs but blending and grinding procedures and a considerable amount of material handling are still present. In addition, the method is not suitable for many drugs which are not readily compressible.

\* Received August 21, 1959, from the laboratories of the Wisconsin Alumni Research Foundation, Madison.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

U. S. Patent applied for.

† Present address: School of Pharmacy, University of Wisconsin, Madison.

The air-suspension granulating method discussed in this paper is an attempt to circumvent some of the disadvantages of the above methods. It is extremely rapid and eliminates the need for separate drying, dry blending, and grinding procedures. As currently used, this method is essentially a particle build-up rather than a particle aggregation process. However, if particle aggregation is desired, it too can be obtained by increasing the humidity in the column.

Previous exploratory work (1, 2) showed that it was possible to apply common granulating materials to small particles and then immediately place the formed granules in a tablet machine without further treatment. Therefore, this paper deals mainly with such aspects as the loss of material during operation and the drug and water concentration in the finished granules rather than a detailed description of the process.

### EXPERIMENTAL

The procedure employed in preparing the granulations was similar to that previously described (1) for the air-suspension coating technique. Solid particles were coated with common granulating materials while suspended in an upwardly moving air stream in a vertical column. The apparatus used in this process is shown in Fig. 1.

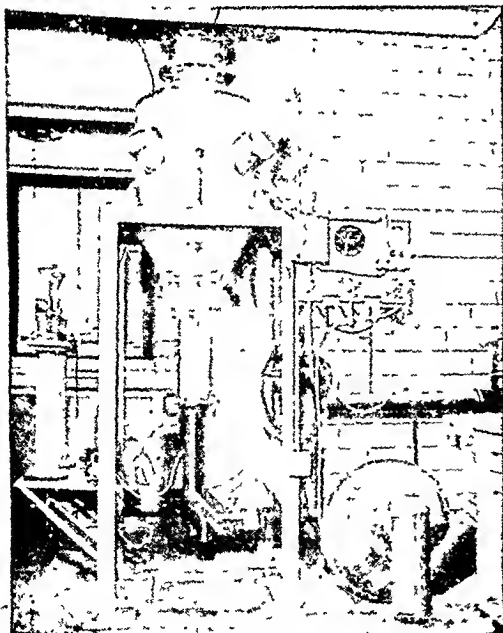


Fig. 1.—Air-suspension granulating apparatus

In this work 3 to 4 Kg. of 30- or 40-mesh particles were introduced into the column and coated with 2 to 22 Kg. of granulating solids. In this case sugar particles served as the nuclei of the resulting granules; however, the nucleus can be composed of either inert material or a drug. When the nucleus is a

drug the granulating materials can be applied to the surface. Conversely, with an inert nucleus the drug can be included in the coat. With the above procedures nearly any drug concentration can be obtained in the granulation.

A variety of granulating materials from both aqueous and organic solvent systems have been applied to solid particles. The coating fluid used in this work was composed of 1,000 Gm. sodium bicarbonate, 496 Gm. lactose, 471 Gm. starch, 8 Gm. sodium lauryl sulfate, 500 ml. of 5% starch paste, and 500 ml. water. The sodium bicarbonate served as a test substance for assay purposes.

Since this investigation was concerned only with the aforementioned quantitative aspects of this process none of the granulations prepared for this series of tests were subjected to the tableting process. Also, no attempt was made to find formulas which would give better bonding to the nucleus or yield satisfactory tablets.

**Operating Conditions.**—In the last four tests shown in Table I an attempt was made to predetermine operating conditions to achieve a 90% humidity in the column. A psychrometric chart was used for this purpose. For example, with wet and dry bulb temperatures of 71° F. and 80° F., respectively, for the air entering the blower, a 140° F. temperature for the inlet air to the column, an air flow of 60 c. f. m. in the column, an 88.5° F. exhaust temperature, and a 90% humidity condition in the column, an approximate drying capacity of 0.0508 lb. of water per minute was obtained. Thus, 3 L. of a coating fluid containing 22 lb. of water was atomized in 43.3 minutes (69.3 ml./min.). For a condition of 80% humidity in the column with an exhaust temperature of 91.5° F., other conditions remaining the same, an approximate drying capacity of 0.0487 lb. of water per minute and an atomization rate of 66.3 ml. per minute were obtained.

**Methods of Analysis.**—The loss of solids was determined from the weight difference between the total solids introduced and the solids recovered from the column. This loss was assumed to be due to the entrainment of fine particles in the exhaust.

The per cent deviation of the sodium bicarbonate content of the finished granulation from the calculated amount was determined with the U. S. P. XV assay method.

The moisture content was determined from the difference between the initial weight of the samples of the granulation and the weight obtained after drying in a vacuum oven at 55° for fourteen hours.

The granulations were classified according to mesh size using standard sieves and a Ro-Tap testing sieve shaker.

### RESULTS AND DISCUSSION

**Loss of Solids.**—In Table I data on the loss of solids, the drug content, and the moisture content are presented. The last four tests were performed with predetermined conditions (90% humidity), as previously described, rather than by a trial and error method. From these data it appeared that the loss of solids increased as the humidity in the column and the moisture content in the finished product decreased. The formation of the fine particles which were lost due to entrainment in the exhaust was prob-



TABLE I.—DATA ON MATERIAL LOSS AND DRUG AND WATER CONCENTRATIONS IN A SERIES OF COMPRESSED TABLET GRANULATIONS

Test No	Mesh Size of Starting Solids	Weight of Total Solids Introduced into Column, Gm	Weight of Solids Recovered from Column, Gm.	Solids Lost, %	Gm. of $\text{NaHCO}_3$ per Gm. of Product Calcd.	Gm. of $\text{NaHCO}_3$ per Gm. of Product Found	Deviation of $\text{NaHCO}_3$ Content, %	Moisture in Granulation, %
1	30	6,000	5,863	2.76	0.1667	0.1632	- 2.09	1.02
2	40	6,000	5,550	7.50	0.1667	0.1378	-17.33	0.54
3	40	6,000	5,845	2.58	0.1667	0.1557	- 6.05	1.18
4	20-30	5,000	4,834	3.32	0.2000	0.1988	- 0.60	1.97
5	10	6,000	5,956	0.88	0.1667	0.1652	- 0.89	3.13
6	30	6,200	6,010	3.06	0.1613	0.1601	- 0.74	1.87
7	40	6,200	5,772	6.09	0.1613	0.1574	- 1.42	1.15
8	30	6,200	6,100	1.66	0.1613	0.1639	+ 1.61	1.98
9	30	6,200	6,094	1.71	0.1613	0.1558	- 3.41	2.49
10	40	6,200	6,089	1.79	0.1613	0.1687	+ 4.58	3.33
11	30	6,200	6,111	1.48	0.1613	0.1593	- 1.23	1.20

ably due to a combination of attritional and spray drying effects at low column humidities. Although these losses can be recovered from the exhaust this was not done in this study. When the exhaust temperature approached that calculated for the dew point, aggregation of particles was observed. The formation of fines is also indicated in Fig. 2 where the particle size distribution of the starting material (approx. 40 mesh) and the two extremes, based on the water content (test 2, 0.54%  $\text{H}_2\text{O}$  and test 10, 3.33%  $\text{H}_2\text{O}$ ) are shown. In test 10, evidence of particle growth is shown whereas in test 2, no appreciable particle growth occurred and the fines increased. It is also pointed out that a considerable amount of material was lost (Table I) which would increase the per cent of fines in test 2.

**Drug Content.**—Assays on the sodium bicarbonate content of the finished granulation tend to indicate that with optimum operating conditions satisfactory drug concentrations can be obtained by this method. Unfortunately, the moisture content for the starting materials was not determined. This may possibly account for the large deviation in the sodium

bicarbonate content (+4.58%) observed in test 10 since these values were corrected for the water content in the finished product. Other large deviations, especially tests 2 and 3, correlate fairly well with the low moisture content of the granulation.

**Water Content.**—Experimental evidence indicates that the water content of the granulation can be controlled within the limits which are satisfactory for the subsequent tableting process. In this series of tests, using vacuum oven drying, the water content varied between 0.54 and 3.33% with varying column conditions. In another series of six tests the water content, as determined with a Cenco moisture balance, varied between 0.9 and 2.2%.

As the ratio of the weight of applied solids to the weight of the nucleus increased, improved flow properties of the solid were noted. For example, when the applied solids on sugar crystals (30 mesh, angle of repose  $36^\circ$ ) in the form of cubes constituted 10% of the final weight, the original crystalline form tended to be maintained. However, when the applied solids constituted 50% of the final weight, the particles tended to become somewhat spherical in shape (angle of repose  $28^\circ$ ) and the flow properties appeared to be enhanced.

A subsequent paper will deal more specifically with the fundamentals of the process and methods of predicting operating conditions for different solvent systems.

## SUMMARY

A new method for preparing compressed tablet granulations by a process similar to the air-suspension coating technique is discussed. Based on the experimental evidence obtained it appears that the described process can be controlled to maintain material losses during the operation of the column and the drug and water content of the finished granulation within reasonable limits.

## REFERENCES

Fig. 2.—A plot of the size distribution of uncoated and coated particles. ○—uncoated particles; × — — — coated particles, test no. 2, 0.54% water; ● — — — coated particles, test no. 10, 3.33% water.

- (1) Wurster, D. E., *THIS JOURNAL*, 48, 151 (1959).
- (2) Wurster, D. E., "Air Suspension and Particle Build-Up," First Annual National Pharmaceutical Research Conference, June 1959, Land O' Lakes, Wis.

# Physical Properties of Lipids Used in Pharmacy I\*

## Screening Raw Materials Via Photomicrography

By DAVIS R. REESE, CLIFFORD W. CHONG, and JOSEPH V. SWINTOSKY

A simple photomicrographic technique has been devised to screen lipids for integrity of their crystal structure. In this technique, thin films of the materials are prepared on microscope slides and photomicrographs are taken of their surface and internal structures. The samples are then stored at normal or exaggerated temperatures for any desired period of time. At various intervals, the exact areas appearing in the original photomicrographs are rephotographed and compared with the originals. These permanent records permit detection of subtle changes which occur during aging. The technique has been used with a number of individual materials and simple mixtures. Representative photomicrographs are presented showing the structural integrity of carnauba wax, cetyl alcohol, hydrogenated soya oil, lauryl monoethanolamide, stearyl alcohol, and white wax.

THIS PAPER describes a photomicrographic procedure for discerning the physical integrity of lipid substances. The procedure is illustrated with data from thin films of carnauba wax, cetyl alcohol, hydrogenated soya oil, lauryl monoethanolamide, stearyl alcohol, and white wax.

Many pharmaceutical dosage forms contain one or more fats or waxes. A number of problems associated with these dosage forms are directly related to changes that occur in their lipid components upon aging. For example, changes may occur which affect the hardness of suppositories, the spreadability of ointments, and the absorption of drug from the dosage form.

A program has been established in our laboratory to study lipid or lipid-containing materials. Emphasis in the program is on the nature and degree of physical change these materials undergo after storage. We have found a photomicrographic procedure especially useful to screen the materials for their physical integrity. This screening procedure is designed to detect lipids which are likely to undergo sufficient physical change to alter the elegance and efficacy of dosage forms derived from them. The basic assumption of the test is that the nature and degree of physical change in thin lipid films can be used in predicting the physical changes these same lipids may undergo in dosage forms.

In our procedure, photomicrographs are taken before and after storing the thin lipid films at different temperatures. Comparisons of identical fields on the photomicrographs reveal crystal transformations, crystal growths, or the development of microscopic cracks.

## EXPERIMENTAL

**Apparatus.**—The basic items needed for micro observations are a polarizing microscope with a graduated mechanical stage, an adequate light source, a camera with a suitable holder, and a photometer. Additional requirements for macro observations are a low magnification system and a suitable light source.

The equipment utilized in our laboratory consisted of a Leitz Panphot camera microscope<sup>1</sup> equipped with polarizing filters and a graduated mechanical stage, a polaroid camera back containing type 44 film,<sup>2</sup> a built-in filament lamp for the micro observations or a ring illuminator for the macro observations, and a Photovolt photometer model 501-M.<sup>3</sup> Three lens systems were used to study the materials included in this communication: Micro system No. 1, periplanatic ocular 10X + apochromatic objective 24/0.65; Micro system No. 2, Huygens ocular 10X + achromatic objective 10/0.25; and Macro system No. 1, Summar lens f 4.5/8 cm. Actual magnification on the photograph for the three systems was 500X, 200X, and 8X, respectively.

**Raw Materials.**—Carnauba wax, yellow Grade 1, E. A. Bromund Co.; cetyl alcohol, N. F., M. Michel and Co., Inc.; hydrogenated soya oil (Textee Flakes), E. F. Drew and Co., Inc.; lauryl monoethanolamide (Kessco X-159), Kessler Chemical Co.; stearyl alcohol, U. S. P., E. I. du Pont de Nemours and Co.; and white wax, U. S. P., Koster-Keunen, Inc.

**Preparation of Slides for Microscopic Observations.**—Thin lipid films, about one crystal thick, make the best samples for microscopic examination.

Crystal changes develop more readily if the films remain uncovered. The films are prepared in the form of wedges to assure the presence of areas approaching optimal thickness. At the time of observation, each wedge is scanned to locate the most desirable field.

Preparation of the slides according to the following procedure has been found satisfactory. First, place a small piece of the test material on a glass slide and

\* Received August 10, 1959 from the Research and Development Division of Smith Kline and French Laboratories, Philadelphia 1, Pa.

The authors wish to acknowledge the assistance of Mr. Peter N. Nordberg in preparing the samples presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

<sup>1</sup> E. Leitz, Inc., New York 16, N. Y.

<sup>2</sup> Polaroid Corp., Cambridge 39, Mass.

<sup>3</sup> Photovolt Corp., New York 16, N. Y.

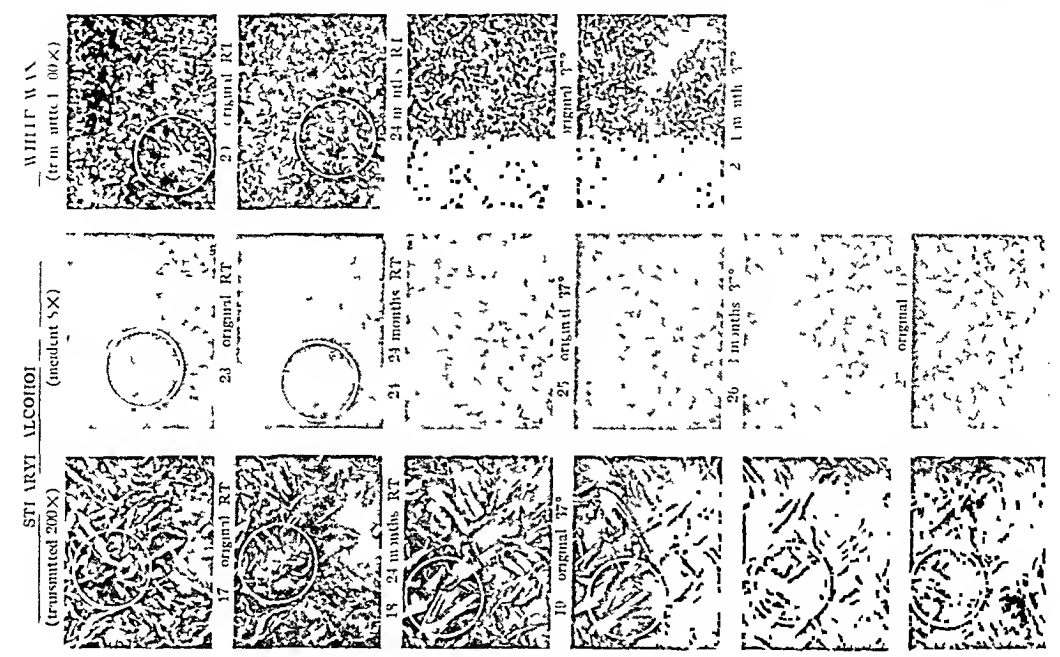
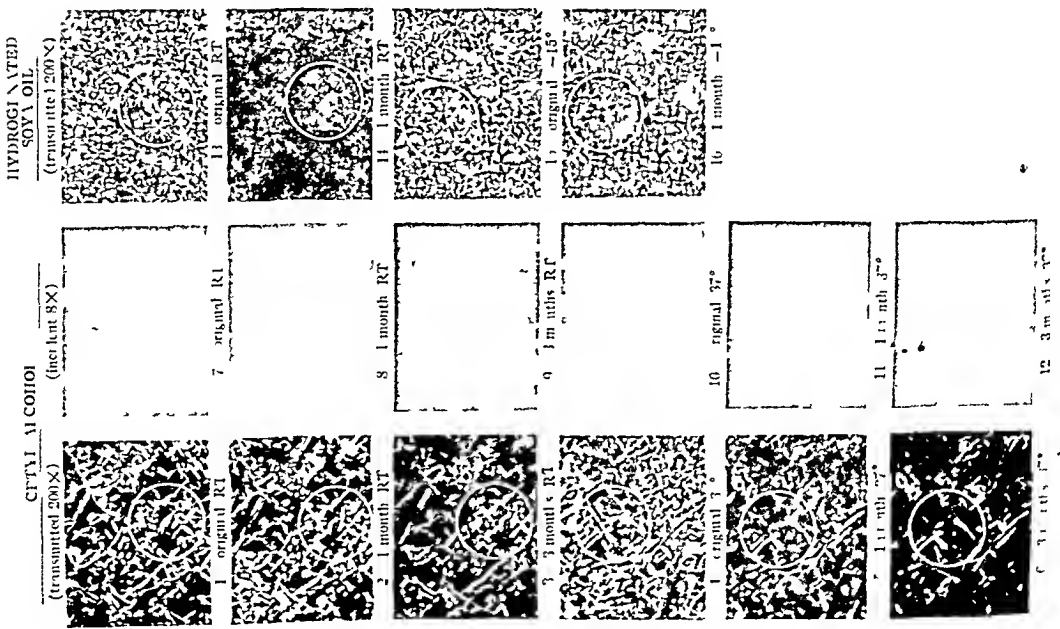


Fig. 1 (left) — Photomicrographs showing the crystal structure of cetyl alcohol and hydrogenated soybean oil (RT denotes room temperature)

Fig. 2 (right) — Photomicrographs showing the crystal structure of stearic alcohol and white wax



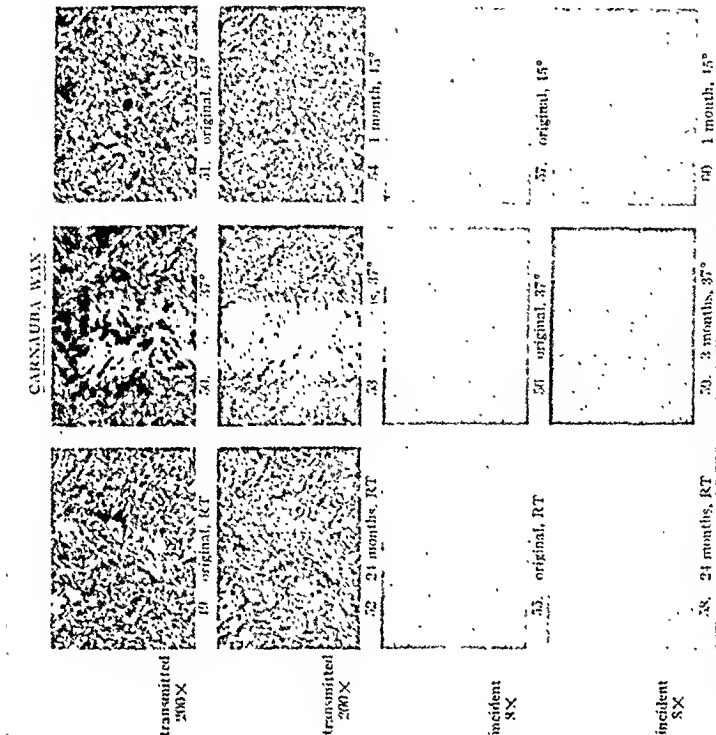
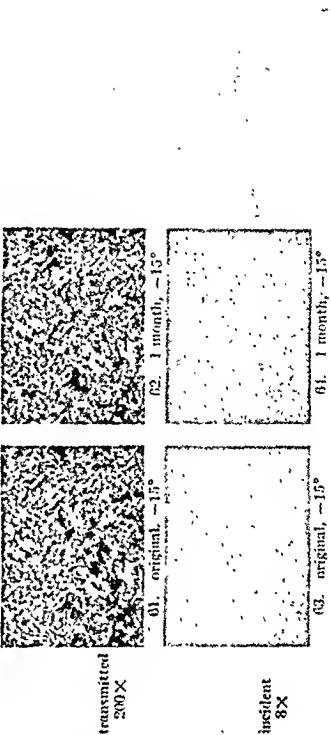


Fig. 3 (left).—Photomicrographs showing the crystal structure of white wax and lauryl monoethanolamide.

Fig. 4 (right).—Photomicrographs showing the crystal structure of carnauba wax.

Note: Some loss of clarity occurred in reproducing the photomicrographs. This is most evident in the photomicrographs taken with incident light, especially Frame 64 (Fig. 4).



heat it gently until melting is complete. Next, spread the molten material into a thin film with a small, warmed glass rod. Then slowly move the rod from one end of the slide to the other until the material begins to solidify. Finally, remove the rod and allow the film of test material to cool. If the slide has been properly prepared, two "wedges" of sample are formed, each tapering from one end of the slide to the center.

At least four such slides were prepared for each material and stored at room temperature for at least two hours before making the original observations. This stabilizing period provided an opportunity for low melting fractions in the sample to crystallize prior to observation.

**Original Observations.**—At the end of the stabilizing period, each slide was observed under the microscope by transmitted polarized light and scanned at several magnifications. When the most appropriate magnification had been selected, a representative area was photographed. The stage settings employed with each photomicrograph were carefully read and recorded. The type ocular and objective used, bellows length, type lighting system, exposure time, and photometer reading were also recorded. A range in magnification from 50–500 $\times$  was used.

When the micro observations were completed, the stage, body tube, and light source were replaced with the macro accessories. The slides were placed on the macro stage which was graduated in  $\frac{1}{4}$  inch units. All macro observations were made by incident light at 8 $\times$ . After taking the photomicrographs the slides were placed in boxes and stored at either  $-15^{\circ}$ , room temperature,  $37^{\circ}$ , or  $45^{\circ}$ .

**Subsequent Observations.**—Reobservations of the slides were made at one week, and at one, three, six, twelve, and twenty four months. The areas to be photographed were located by using the stage readings from the original observation and, when necessary, the slides were further centered by comparing the field in the microscope with the original photomicrograph of that slide. The slides were always scanned to detect significant changes that might have occurred in isolated areas. When any such changes were detected, they were photographed and rechecked in later observations.

Observations were discontinued whenever the room temperature slides exhibited a gross change in crystal structure. All slides stored at exaggerated conditions were transferred to room temperature at the end of three months.

## RESULTS

It is important to note that the samples were obtained by slowly solidifying the melt. Other methods of sample preparation might have yielded different results. Unless otherwise noted, samples stored at  $-15^{\circ}$  showed no change.

**Cetyl Alcohol.**—Frames 1–6 (Fig 1) depict cetyl alcohol magnified 200 times and observed with transmitted light. Corresponding areas within each frame are enclosed by the white circles. Discernable changes occurred after only one month of storage at room temperature (Frame 2). Even more drastic changes occurred at  $37^{\circ}$  (Frames 5 and 6). Frames 7–12 (Fig 1) are incident light observations of the

same slides but magnified only 8 times. The crystal changes are much less evident at the lower magnification. Although these structural changes are in visible to the unaided eye, the data indicate that cetyl alcohol might not be suitable for uses where crystal integrity is a requirement.

**Hydrogenated Soya Oil.**—Transmitted light observations of this material are shown in Frames 13–16 (Fig 1). Although the material has a relatively high melting point of  $67^{\circ}$ , Frame 14 shows that it undergoes severe crystalline change after storage at room temperature for one month. Some change may be noted even after storage at  $-15^{\circ}$  (Frame 16). As with cetyl alcohol, one would be dubious of using this type of hydrogenated soya oil if crystal integrity is important.

**Stearyl Alcohol.**—Frames 16–22 (Fig 2) depict transmitted light observations made after storage at room temperature for twenty-four months, at  $37^{\circ}$  for three months, and at  $45^{\circ}$  for three months. Only minor structural changes have occurred under these conditions. Although stearyl alcohol is closely related to the cetyl alcohol shown in Fig 1, the integrity of its crystal structure is definitely better at the temperatures studied. Frames 23–28 (Fig 2) show that very little change has occurred in the gross structure of stearyl alcohol. From this study, stearyl alcohol would be considered to have sufficient integrity to permit its evaluation in dosage forms requiring a material of high structural integrity.

**White Wax.**—White wax is reasonably stable for twenty four months at room temperature (Frames 29 and 30) (Fig 2). Frame 32 (Fig 2) made after storage at  $37^{\circ}$ , shows several dark areas just below the white arrow. These are thick crystalline areas which have developed. They are dark because light is no longer transmitted through them. The change occurring at  $37^{\circ}$  is much more clearly shown in Frame 36 (Fig 3), which is the incident light observation of the same slide. Numerous crystalline areas cover the surface of the slide. These observations indicate that white wax will maintain satisfactory crystal integrity if the storage temperature is approximately  $25^{\circ}$ . They also indicate that changes occurring at elevated temperatures do not always occur at room temperature within a two year period.

**Lauryl Monoethanolamide.**—This material undergoes some structural change when stored for twenty-four months at room temperature or for one month at  $37^{\circ}$  (Frames 37–41) (Fig 3). Frame 42 (Fig 3) shows that a gross change occurs between one and three months at  $37^{\circ}$  with disappearance of the characteristic structure. The incident light observations of Frames 43–48 (Fig 3) confirm this, but the degree of change is not so apparent. The white area appearing under the arrow on Frame 44 is the result of damage during handling and should be ignored. These observations indicate that lauryl monoethanolamide can probably be used in dosage forms if structural integrity is not highly critical. Prolonged storage at elevated temperatures should be avoided.

**Carnauba Wax.**—Frames 49–54 (Fig 4) show that carnauba wax exhibited very little change in crystal structure when stored at room temperature for twenty four months,  $37^{\circ}$  for three months, or  $45^{\circ}$  for one month. This integrity of the crystal struc-

ure is further exhibited in the incident light observations, Frames 55-60 (Fig 1). From this data, one would conclude that carnauba wax has properties which may make it valuable for pharmaceutical use. Frames 61-64 (Fig 4), however, show that carnauba wax is cracked when stored at  $-15^{\circ}$ . At high magnification (Frame 62) only a few cracks are visible. Two of these are marked with arrows; the crack on the right being partially covered by the arrow. The clarity of the cracking in Frame 64 made with low magnification shows how the incident light observations often supplement those made with transmitted light. These observations indicate that carnauba wax will maintain its crystal structure at room temperature and at somewhat elevated temperatures. Dosage forms employing it may need to be plasticized when they will be exposed to freezing temperatures.

## DISCUSSION

The Panphot camera microscope has several features which we found advantageous. It has a built-in light source, separate oculars for photographic and visual observations, an attached camera with viewing screen, and it can also be used for both macro and micro observations.

The use of Polaroid film permitted rapid development of photomicrographs representing original observations and also eliminated the need for a photographic darkroom. The rapid availability of the photomicrographs permitted the defective ones to be remade prior to storage of the slides at evaporated temperatures.

Since the area photographed changes inversely with the magnification, total magnification should be just sufficient to permit distinct observation of a characteristic structure. Choice of proper magnification for the micro and macro observations cannot be overemphasized. The micro observations were made with transmitted light to detect minute structural changes. The usual magnification for these observations was 50-200 $\times$ , but occasionally ran as high as 500 $\times$ . Magnifications of the photomicrographs used in this communication are sometimes greater than found necessary in practical screening studies. These were employed to enhance clarity in the reproductions of the photomicrographs.

The macro observations were made with incident light to detect gross surface changes which might have an adverse effect on the elegance of pharmaceutical products. Optimum magnification was usually 5-10 $\times$ . The ability to pick up gross changes at these lower magnifications was clearly shown in the cracked sample of carnauba wax (Fig 4).

The photomicrographic technique has several features which make it useful. (A) thin films of samples are employed to give maximum sensitivity in detection of structural change, (B) for reobservations

of stored slides, the exact areas observed in the original photomicrographs are used, (C) the photomicrographs are permanent and they can be studied whenever desired, and (D) the consecutive photomicrographs of a sample facilitate the determination of the approximate rate and severity of structural change. On the other hand, the method has three disadvantages. (A) in dosage forms, the structural integrity of the lipid components may be affected by the other components. Therefore, films of single lipids may not always reflect the changes these lipids would undergo when blended with other lipids or pharmaceutical agents, (B) the causes of the crystal changes are difficult to interpret, and (C) the integrity of the sample following storage cannot be predicted from the original observation.

Crystal transformations in fats and waxes are generally the result of polymorphism, i.e., the ability to exist in more than one crystalline form (1-3). In a polymorphic substance the crystal form present at any given time will depend in part on the heat history of the sample. During storage, if a transition occurs, it is normally toward a more stable crystalline form. Crystal size, melting point, solubility, and other properties may be quite different from those of the original forms. Thus, these new forms, although more stable, may be detrimental to the appearance and desired performance of a manufactured product.

Heterogeneous lipid materials may also show a change in solid-liquid ratio with a change in storage temperature and/or with the passage of time. An increase in solid content occurs as entrapped liquid gradually crystallizes into solid forms, whereas a decrease in solid content occurs when the temperature is elevated above the melting point of low melting fractions in the lipid.

Although the data reported here are limited to several individual lipids, the photomicrographic technique has potential use with lipid mixtures and with all drugs or dosage forms which may undergo crystalline changes.

Among the potential applications for the technique in lipid dosage forms are (A) to determine the maximum amount of a specific lipid raw material which could be used without causing adverse structural effects, (B) to determine the effect on lipid structural integrity of introducing a drug into a master formula, and (C) to compare the structural integrity of several alternate development forms of a product.

## REFERENCES

- (1) Bailey, A. E. *Melting and Solidification of Fats*. Interscience Publishers Inc. New York, N. Y., 1950.
- (2) Holman, R. T., Lundberg, W. O. and Malkin, T., editors. *Progress in the Chemistry of Fats and Other Lipids*. Vols. I and II. Academic Press Inc. New York, N. Y. 1952 (Vol. I) and 1954 (Vol. II).
- (3) McCrone, W. C. Jr. *Fusion Methods in Chemical Microscopy*. Interscience Publishers Inc. New York, N. Y. 1957, p. 132.

# The Biological Action of Cellular Depressants and Stimulants IV\*

## The Effect of Phenylurethane on the Onset and the Magnitude of Synchronous Cell Division of *Tetrahymena pyriformis* GL

By WALTER SINGER†, KWAN-HUA LEE, and JOHN J. EILER

Phenylurethane is shown to delay the onset of synchronous division and to decrease the maximum division index in cultures of *Tetrahymena pyriformis* GL synchronized by temperature cycling.

A FIRST APPROACH to understanding how an agent exerts a narcotic action upon cell processes is to measure the effect of the narcotic upon physiological and cytological characteristics of cells. Unicellular microorganisms have often been utilized for such studies. An actively growing population of microorganisms is a heterogeneous mixture of cells representing all stages of a life cycle in which an individual cell divides to form two cells, each of which develops over a span of time into a mature cell which again divides. Recently developed synchronization techniques (1, 2) can produce populations of bacteria, yeast, or protozoa in which most of the cells have been brought into the same phase of the life cycle. Large homogeneous masses of cells are thereby made available for study.

Scherbaum and Zeuthen (3-5) have described a method for synchronizing the ciliate protozoan, *Tetrahymena pyriformis*, by exposure of an actively growing log-phase culture to brief periods of sublethal temperature shocks alternated with exposure to equal periods at a lower temperature optimal for growth. During this temperature cycling, the cells increase in mass but do not divide. When finally returned to, and kept at optimum temperature, the cells grow slightly and begin to divide only after a quite constant lapse of time. After approximately eighty minutes at optimum temperature, about 80 per cent of the cells are seen to be undergoing fission. This essentially simultaneous entry into the phase of division is the evidence that the tetrahymena have been syn-

chronized by the heat treatment. Subsequently, a second and a third synchronous division may be observed.

As a part of a continuing interest in the mechanism of narcotic action, workers in this laboratory have studied (6, 7) the effect of urethane on the growth and respiration of asynchronously growing cultures of *Tetrahymena pyriformis* W and on the oxidative phosphorylation of cell-free preparations (8). The results obtained indicate that the action of urethane to decrease the rate of cell division of tetrahymena is associated with a reduction of an oxygen-dependent growth factor. The system devised for synchronizing tetrahymena offers an opportunity to extend the observations of the effects of members of the urethane series to (a) tetrahymena being synchronized by temperature cycling and to (b) the resultant synchronized cells. Such a study deals, in the first instance, with cells that are growing but not able to divide and, in the second, with cells that are all in the same phase of their life cycle and soon destined to enter simultaneously into the process of cell fission. Accordingly, this investigation was concerned with the effects of phenylurethane, a potent narcotic, upon significant biological parameters related to growth and cell division in cultures of tetrahymena treated to produce synchronous cell division. This is a report of the action of phenylurethane to delay the onset of synchronous division and to decrease its magnitude. Effects of this agent upon cell volume increase and upon the synthesis of protein and desoxyribonucleic acid during and after heat treatment are reported separately (9).

### MATERIALS AND METHODS

**Culture Techniques.**—Axenic stock cultures of *Tetrahymena pyriformis* GL (obtained through the courtesy of G. W. Kidder) were maintained in slanted test tubes at 28° in 1.8% proteose-peptone (Difco) plus 0.2% yeast extract (Difco). Cells ( $0.6$  to  $15 \times 10^5$  in number) from a forty-eight hour old stock culture were inoculated into 400 ml. of sterile culture medium in a 2-L. Erlenmeyer flask

\* Received August 21, 1959, from the School of Pharmacy, University of California, San Francisco 22.

This paper is adapted from the prize-winning manuscript submitted by Walter Singer in the 1958 Lunsford Richardson Pharmacy Awards competition.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Fellow of the American Foundation for Pharmaceutical Education, 1955-1956, and Smith, Kline and French Fellow, 1956.

and allowed to multiply for twelve to twenty-four hours to provide a sufficient quantity of organisms for each experiment

**Synchronization Procedure.**—The synchronization procedure was patterned after that developed by Scherbaum and Zenthen (2-5). Twenty-five milliliter suspensions of logarithmically growing cultures in proteose peptone and yeast extract medium ( $0.2$  to  $2.1 \times 10^6$  cells per ml) were aseptically pipetted into sterile 300 ml Erlenmeyer flasks, usually six in number. The flasks were suspended in a water bath fitted with controls (10) which could be set to alternate the water temperature between two levels. The cells were exposed to half-hour periods at  $34^\circ$  alternated with half hour periods at  $28.2^\circ$ . It required eight minutes to raise the bath temperature from  $28.2$  to  $34^\circ$  and twelve minutes to lower the temperature again. The eight minutes were included in the half hour at  $34^\circ$ , the twelve minutes were included in the half hour at  $28.2^\circ$ . After the eighth period at  $34^\circ$ , the bath temperature was allowed to drop to  $28.2^\circ$  and was then kept constant at this level. The flasks were shaken throughout the treatment. With this procedure, a good first synchronous division was always obtained and a second was sometimes seen. A third synchronous division was not observed to occur.

**Cell Counting.**—One-milliliter samples were pipetted from test cultures at appropriate intervals. The cells were killed by the addition of 0.3 ml of formalin. The number of cells per ml of sample was determined using a Sedgewick Rafter counting chamber and a Whipple micrometer (6, 11, 12). A second count of only the number of dividing cells was also made for each sample. Any cell showing any degree of cytoplasmic fission was considered to be a dividing cell. A 5 power ocular and 16-mm objective were used. Photomicrographs were taken of key samples.

**Reagents.**—Phenylurethane (Eastman)  $C_6H_5-NH-COOC_2H_5$ , recrystallized once from petroleum ether, was dissolved in 95% ethanol to prepare stock solutions such that 0.05 ml added to 25 ml of cell suspension would provide the desired final drug concentration.

## RESULTS AND DISCUSSION

One measure of the degree of synchronization is the division index (4) which is defined as the ratio of the number of dividing cells to the total number of cells at a given instant. The maximum division index, i.e., the highest such ratio found during the time interval through which the synchronous division takes place, is a most useful criterion of synchronization. Its magnitude and the time at which it occurs can be readily accurately determined by a graphical method using values for division indexes from the several samples taken at intervals during a synchronous division outburst (see Figs 1 and 2). Under the experimental conditions used, the value of the maximum division index and the length of the lag period (the interval between the end of temperature cycling and the occurrence of the maximum division index) were found to be quite reproducible for first synchronous division.

The effect of phenylurethane on these parameters

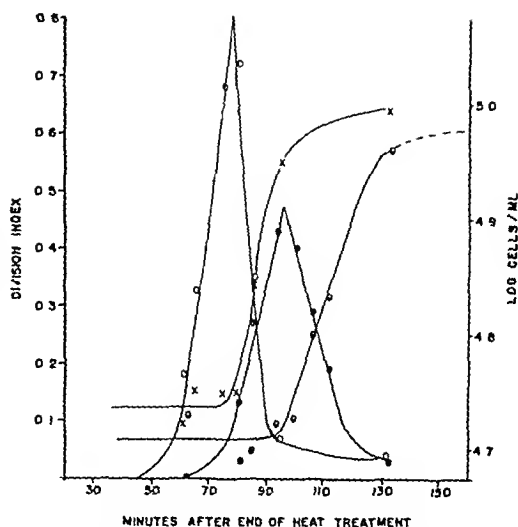


Fig 1—The effect of phenylurethane on division index and on population density.  $6 \times 10^{-4} M$  phenylurethane added before heat treatment. Division index,  $\circ$  = control,  $\bullet$  = drugged. Population density,  $\times$  = control,  $\circ$  = drugged.

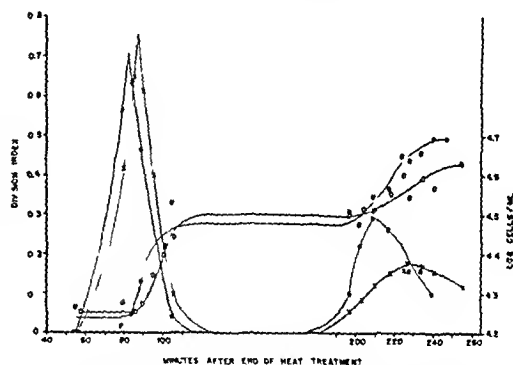


Fig 2—The effect of phenylurethane on division index and on population density.  $6 \times 10^{-4} M$  phenylurethane added after heat treatment. Division index,  $\bullet$  = control,  $\circ$  = drugged. Population density,  $\times$  = control,  $\circ$  = drugged.

of synchronous division and upon increases in cell number was studied under two general conditions, i.e., when the drug was added just before starting the heat treatment and when it was added approximately fifteen minutes after the final  $34^\circ$  level. In either case, 0.05 ml of the drug in 95% ethanol was added aseptically to 25 ml of cell suspension to yield a final concentration of  $6 \times 10^{-4} M$  with respect to phenylurethane. This concentration has been shown by conventional turbidity measurements to inhibit reversibly the total cell mass increase (by 50%) in asynchronous cultures of tetrahymena; it also decreases the rate of cell multiplication in these cultures as measured by cell count (unpublished data).

**The Effect of Phenylurethane on First Synchronous Division:  $6 \times 10^{-4} M$  Phenylurethane Added Before Heat Treatment.**—The effects of phenylurethane on the first synchronous division of cells which were synchronized by heat treatment in the presence of the drug are shown by data in



TABLE I.—THE EFFECT OF PHENYLURETHANE ON FIRST SYNCHRONOUS DIVISION

	Max. Index	Time <sup>a</sup>	Log Phase	Cells/ml. × 10 <sup>-3</sup> Prediv.	Postdiv.	Post-/Pre-
Controls <sup>b</sup>	0.77 ± 0.01	80 ± 0.7	47.7	54.4	95.1	1.7 ± .03
Drugged <sup>b</sup>	0.42 ± 0.02	100 ± 0.9	47.7	53.3	79.9	1.5 ± .04
Controls <sup>c</sup>	0.69 ± 0.02	83 ± 1.6	..	..	..	....
Drugged <sup>c</sup>	0.61 ± 0.03	89 ± 1.8	..	..	..	....

<sup>a</sup> The number of minutes between the end of the heat treatment and the attainment of the maximum division index.  
<sup>b</sup> Average values for 14 experiments in which  $6 \times 10^{-4}$  M phenylurethane was added before heat treatment.  
<sup>c</sup> Average values for 11 experiments in which  $6 \times 10^{-4}$  M phenylurethane was added after heat treatment.

The standard errors shown here were calculated from the formula:  $S. E. = \pm \sqrt{\frac{\sum(X - \bar{X})^2}{n(n - 1)}}$

Table I and in Fig. 1. It should also be brought out here that, during the heat treatment, the control cells almost tripled in size and in protein content relative to log-phase cells, and that the drugged cells increased only to about three-fourths of the size of the control cells (9). One effect is a decrease in the magnitude of the maximum division index; the average value for drugged cells was 54.5% of that for the controls. A second is a twenty-minute increase in the time interval between the end of the heat treatment and the attainment of the maximum division index. Figure 1 is a graphical presentation of these effects as observed in a typical experiment. It should also be noted that the delay in reaching the maximum division index was accompanied by a delay in the onset of division. The delay in onset, although clearly evident (Fig. 1), was not amenable to quantitative treatment because it was difficult to pinpoint the time at which the first cytoplasmic furrowing occurred.

A third effect of the drug, when added prior to heat treatment, is a decrease in the number of cells resulting from the first synchronous division. In Table I, the population densities of control and of drugged cultures are compared. The predivision density is the average number of cells per ml. in the three or four samples taken before peak division index; this represents the cell population at the end of the heat treatment. Predivision population densities of drugged cultures did not differ significantly from those of the controls. Each postdivision density was obtained from a single sample collected when the first synchronous division outburst was over. Table I shows that the postdivision population density of drugged cultures averaged 84% of that of the controls. The 1.5-fold increase in drugged cell count during the synchronous division is significantly lower ( $p = 0.01$ ) than the 1.7-fold increase in control cell count.

A fourth drug effect is an increase in the time needed to complete the first synchronous division outburst once it has started. In Fig. 1 it can be seen that the rate of change in division indexes was less rapid in the drugged culture than in the control. Also it is apparent in Fig. 1 that the cell population in the drugged culture increased less rapidly than in the control.

The lesser slopes of these temporal changes in division indexes and in population increases suggested that phenylurethane might be adversely affecting the induced phasing of the tetrahymena. Evidence for this point of view was obtained from a differential count of fission stages. This count was made from photomicrographs taken of cell suspensions which were just before or just after peak divi-

sion. Five categories of cells were set up, namely, (a) not divided, (b) starting to divide, (c) half-way divided, (d) nearly split apart, and (e) daughter cells. The decision to place a cell into a specific category had to be based upon a close scrutiny of the outline of the fixed cell followed by an estimation of how far along in the fission process the cell had progressed. Some inaccuracy must necessarily result from attempting to divide into discontinuous groups a continuous action such as cell fission.

The data are summarized in Fig. 3. For the control culture, the finding of the definite high per cent peak at progressively later fission stages as the time of maximum division index was approached and passed, shows that a large group of these cells began to divide at the same time and continued to pass as a group through the entire fission process. This is the picture to be expected for good synchrony.

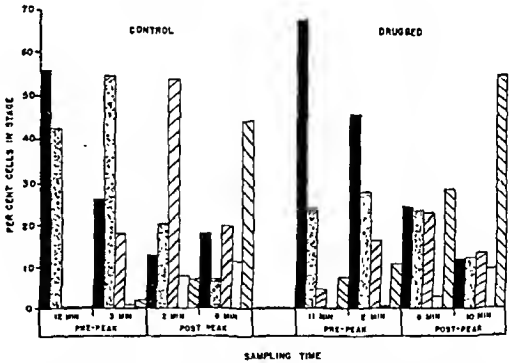


Fig. 3.—The effect of phenylurethane on the distribution of fission stages of Tetrahymena during first synchronous division:  $6 \times 10^{-4}$  M phenylurethane added before heat treatment. Approximately 2,500 drugged and 2,500 control cells were cataloged. ■ = not divided; ▨ = starting to divide; ▩ = half-way divided; ▪ = nearly split apart; □ = daughter cells.

The drugged cells did not show this formation of a well-defined group. Large groups were found only at two times, i. e. at eleven minutes pre-peak when most of the cells had not yet begun to divide and again at ten minutes postpeak when daughter cells were in the majority. Near the time of the maximum division index, especially shortly postpeak, the cells were about evenly proportioned among four stages. This is in sharp contrast to the findings in the control culture and indicates that phenylurethane had interfered with the phasing of the cells.

Each of the effects on the parameters of first syn-

chronous division which are observed when phenylurethane is added before heat treatment can be attributed to inhibitory actions of the drug on a cellular system or systems concerned with division. The delayed onset of division is direct evidence that phenylurethane either slows formation of material essential to initiation of division or else prevents for some time the action of the material once formed. The out-phasing of the cells manifested as a decreased rate of change of division indexes and of population can arise from a randomized entry into the division phase due to individual variation in cell susceptibility to phenylurethane inhibition. Some cells are affected by the drug to such an extent that they fail to divide during the synchronous division outburst. There would appear to be a relationship between the failure of a proportion of the drugged cells to divide during the first synchronous division and the observed lowered value for the maximum division index of the drugged cells. The drugged cell population was found to average 84% of that of the control cells after completion of the division outbursts (Table I). From the maximum division indexes (Table I), one can calculate that the ratio of the number of drugged cells to control cells after synchronous division would be  $1.42/1.77$ , i. e., that the drugged cell population would be 80% of that of the control cells. The correspondence between these figures indicates that the lowered division index reflects an action of phenylurethane to prevent the division of a number of cells. Possibly the out-phasing of the cells also contributes to lowering the maximum division index.

Data in Table I provide evidence that cells may reach a stage in the fission process which is insensitive to inhibition by phenylurethane. The small increase in cell numbers which occurred during the heat treatment is taken to indicate that temperature cycling did not stop the division of cells which had already begun to divide (5). Since the drugged cell and the control cell populations increased equally during heat treatment, apparently phenylurethane also did not stop division once it had actually started.

**Effect of Phenylurethane on First and Second Synchronous Division:**  $6 \times 10^{-4}$  M Phenylurethane Added After Heat Treatment.—When phenylurethane was added to the culture medium of synchronized cells at approximately fifteen minutes after the end of the final  $34^\circ$  heat treatment, two effects on the division index of the first synchronous division were noted. These were qualitatively but not quantitatively the same as those found when the phenylurethane was added prior to synchronization. The division index maximum for the drugged cells was lower and it was reached later. However, as shown by the data in Table I and Fig. 2, the effect of the phenylurethane added after synchronization was less marked than that produced by adding it prior to synchronization. Although the division index maximum was lowered in 9 out of 11 experiments, the effect was pronounced in only one experiment. In five experiments, the drugged cell maximum index was between 80 and 90% of that of the controls; in three others it was more than 90% of the control value and, in two experiments, drugged cell division index maxima were slightly higher than the control values. The average drugged cell maximum divi-

sion index was 88.4% of the control value. In these same experiments, the drugged cells reached maximum division index, on the average, six minutes later than did the control cells. The delay, observed in 10 of the 11 experiments, is statistically significant ( $p = 0.05$ ).

When phenylurethane was added after heat treatment, it showed little ability to prevent cells from dividing during first synchronous division. Post-division population data are not available because the taking of samples was terminated in a number of these experiments before the drugged cell synchronous division was completely over. However, when data from individual experiments are plotted as in Fig. 2, it can be seen that exposure to phenylurethane only during the lag period did not consistently decrease the number of cells present at the end of first synchronous division. This is in contrast to the decrease produced when the drug was added before heat treatment.

In two experiments in which phenylurethane was added after heat treatment, data for a second synchronous division were obtained. Figure 2 includes these data for one experiment. The control cells were no longer well phased. This was shown by the lower maximum division index (0.28), by the increased spread of the division index curve along the time axis, and by the lesser slope of the population density curve when compared to the similar features of the first synchronous division. The phenylurethane, which had been in contact with the cells since shortly after the conclusion of the heat treatment (about three and one-half hours), exaggerated each of these changes. When compared with the controls, the maximum division index of the drugged cells was lower (0.18), the spread of the division index curve along the time axis is further widened, and the slope of the population density curve is clearly lessened. In addition, the drugged cell maximum index occurred twenty-one minutes later than did that of the controls; the delay was only five minutes during the first synchronous division. It is also evident that at the end of the second synchronous division the drugged cell population density was less than that of the controls.

Thus, phenylurethane added after the synchronizing heat treatment is completed exerts more pronounced effects upon the second synchronous division than upon the first. In fact, the effects upon second synchronous division are quantitatively quite similar to those which phenylurethane added before heat treatment has upon the subsequent first synchronous division.

Synthesis of cellular material is a characteristic common both to the period of heat treatment and to the period between the first and the second synchronous division. There appears to be relatively little synthesis of protein during the first lag period (9, 13); DNA may (14) or may not (9) be synthesized. Phenylurethane shows its greatest effect on synchronous divisions which follow contact of the drug with tetrahymena during a time of active growth. The drug does not prevent cell fission once it has started. The evidence is, therefore, that the effects of phenylurethane reported herein are a consequence of its overall inhibition of cellular synthesis which slows or prevents formation of a material essential to initiation of fission.

## SUMMARY

1. A narcotic concentration of phenylurethane is shown to delay the onset of synchronous division and to reduce the maximum division index in cultures of *Tetrahymena pyriformis* GL synchronized by temperature cycling.

2. Phenylurethane effects are more pronounced when the drug is present during the temperature cycling than when the drug is added after the cycling.

3. The delay in onset of division is attributed to a decreased rate of production of material necessary for cell division. Drug-induced randomization of the entry of cells into division is a reflection of the varied susceptibility of the cells. The lowered maximum division index

results from failure of some of the more susceptible cells to divide.

## REFERENCES

- (1) Campbell, A., *Bacterial. Rev.*, 21, 263(1957).
- (2) Zeuthen, E., *Advances in Biol. and Med. Phys.*, 6, 37(1958).
- (3) Scherbaum, O., and Zeuthen, E., *Exptl. Cell Research Suppl.*, 3, 312(1955).
- (4) Zeuthen, E., and Scherbaum, O., *Prac. Symposium Calston Research Soc.*, 7, 141(1954).
- (5) Scherbaum, O., and Zeuthen, E., *Exptl. Cell Research*, 6, 221(1954).
- (6) Eiler, J. J., Krezanoski, J. Z., and Lee, K. H., *THIS JOURNAL*, 48, 200(1959).
- (7) Eiler, J. J., Krezanoski, J. Z., and Lee, K. H., *ibid* 48, 666(1959).
- (8) Krezanoski, J. Z., Eiler, J. J., and Lee, K. H., to be published.
- (9) Singer, W., and Eiler, J. J., in press.
- (10) Lee, K. H., *THIS JOURNAL*, 48, 468(1959).
- (11) Hall, R. P., Johnson, D. F., and Loefer, J. B., *Trans. Am. Microscop. Soc.*, 54, 218(1935).
- (12) Scherbaum, O., *Acta Pathol. Microbial. Scand.*, 40, 7(1957).
- (13) Christensson, E., *Acta Physiol. Scand.*, 45, 339(1959).
- (14) Scherbaum, O., *Exptl. Cell Research*, 13, 24(1957).

## The Development of a Liquid Antihistaminic Preparation With Sustained Release Properties\*

By HARRY A. SMITH†, ROBERT V. EVANSON, and GLEN J. SPERANDIO

The relative capacity of several cation exchange resins to adsorb methapyrilene has been determined by an *in vitro* method. It was demonstrated that methapyrilene adsorbed on sulfonic acid cation exchange resin possessed pharmacological activity approximating that of a solution of methapyrilene hydrochloride as measured by the protection against histamine-induced asthma in the intact guinea pig. It is suggested that the use of ion exchange resins as carriers of drugs in liquid dosage forms may be useful in sustained release preparations.

THE COMMERCIAL USES of ion exchange resins have steadily increased since the first synthetic ion exchange resin was prepared by Adams and Holmes in 1935 (1), and the number of publications per year pertaining to ion exchange almost doubled from 1950 to 1955 (2). The use of ion exchange materials in medicine is as old as the medical profession itself (3), a good example being the use of kaolin and similar substances as enteric adsorbents of toxins. The use of synthetic ion exchange resins as medicinal agents has been reported in the medical literature (4-6).

The literature (7-13) indicates that the princi-

ple of sustained release of a drug from its resin-adsorbate might well be applied to orally administered suspensions of the drug. The chief objective of this project was to develop a liquid antihistaminic with sustained release properties.

Although there are available long acting antihistamines, the availability of a sustained release antihistamine in a liquid formulation would be of value in cases where solid medication cannot be taken. Moreover, the demonstration of the feasibility of using cation exchange resins to obtain a controlled, sustained release of drugs in a liquid form was a major consideration.

## EXPERIMENTAL

**Capacity Studies.**—Methapyrilene was chosen as the antihistamine for study and the following cation exchange resins were screened for their capacity to adsorb methapyrilene using the batch technique: Dowex 50<sup>1</sup> resins with 4, 8, and 12% cross-linkage, each with 50-100, 100-200, and 200-400-mesh ranges; and Amberlite IRC 50<sup>2</sup> resins with a 4-6% cross-linkage and 16-50, 100-200-mesh ranges, and 90% passing through a 200-mesh sieve were used in this work. Cross-linkage refers to the internal porosity of the molecular resin structure and is determined by the amount of divinylbenzene added during the styrene-divinylbenzene copolymerization. All of the resins were used in the acid

\* Received April 8, 1959, from the School of Pharmacy, Purdue University, Lafayette, Ind.

Abstracted from a dissertation submitted to the Graduate School of Purdue University by Harry A. Smith in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Fellow of the American Foundation for Pharmaceutical Education. Present address: University of Kentucky, College of Pharmacy, Lexington.

Presented to the Section on Practical Pharmacy, A. Ph. A., Cincinnati meeting, August 1959.

<sup>1</sup> Dowex 50 is the trade name of Dow Chemical Co. for their series of sulfonic acid cation exchange resins.

<sup>2</sup> Amberlite IRC is the trade name of Rohm and Haas Co. for their series of carboxylic acid cation exchange resins.

form. For a comparative study the resins were employed on a dry weight basis, since the various resins adsorb varying percentages of water. The moisture content of each resin was determined with the Cenco moisture balance just prior to each capacity determination.

The batch technique was employed to determine the relative capacity of the cation exchange resins to adsorb methapyrilene. One-gram samples of the resins were mixed with 50 ml. of a 0.1M solution of methapyrilene hydrochloride and agitated until apparent equilibrium was reached. This was determined by analyzing for the unadsorbed methapyrilene spectrophotometrically, using the method of Martin and Harrison (14). When there was no significant change in the amount of unadsorbed methapyrilene, apparent equilibrium was assumed to be established. The amount of methapyrilene adsorbed was calculated as the difference between the amount of methapyrilene hydrochloride in the original solutions and the amount of unadsorbed methapyrilene hydrochloride.

Preliminary experiments were conducted to determine the effect of the concentration of methapyrilene hydrochloride on the amount of methapyrilene adsorbed by a sulfonic acid cation exchange resin with a 4% cross-linkage and a 50-100-mesh range. The results are shown in Fig. 1. A study of these data led to the selection of a 0.1M solution of methapyrilene hydrochloride for further capacity studies since that was the lowest concentration to permit differentiation among the several resins for their capacity to adsorb methapyrilene.

A comparative study was made of the capacity of several sulfonic acid cation exchange resins with various degrees of cross-linkage and particle sizes to adsorb methapyrilene from 50 ml. of a 0.1M solution. The data are shown in Table I.

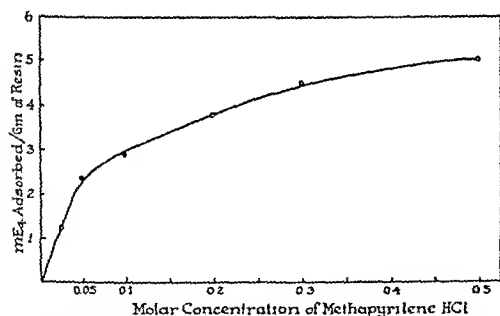


Fig. 1.—The effect of concentration on the amount of methapyrilene adsorbed from solution by Dowex 50, 4% cross-linkage 50-100 mesh.

TABLE I.—THE EFFECT OF CROSS-LINKAGE AND PARTICLE SIZE ON THE ADSORPTION OF METHAPYRILENE BY SULFONIC ACID RESINS

Mesh Range of Resin	meq. of Drug Adsorbed/Gm. of Resin at Apparent Equilibrium		
	4% Cross-Linkage <sup>a</sup>	8% Cross-Linkage <sup>a</sup>	12% Cross-Linkage <sup>a</sup>
50-100	2.71	2.66	2.30
100-200	2.64	2.55	2.31
200-400	2.71	2.65	2.08

<sup>a</sup> Values are the average of three samples.

The results show that adsorption of methapyrilene decreases with an increase in the degree of cross-linkage of the resins. Also particle size has no apparent effect on the amount of adsorption of the drug at apparent equilibrium. However, it was observed that with the 12% cross-linkage, the rate of adsorption was greater with the smaller particle sizes.

Similar experiments were conducted with the carboxylic acid cation exchange resins, except that the adsorption process was permitted to proceed for only three hours for a comparative study. The quantities of methapyrilene adsorbed, expressed in milliequivalents per gram of dry resin, were 1.37 for the 16-50 mesh, 1.37 for the 100-200 mesh and 1.65 for the 200-400 mesh.

It was found that the sulfonic acid resins exhibited a greater relative capacity for adsorbing methapyrilene than the carboxylic acid resins under the conditions of these experiments. However, all of the resins adsorbed a sufficient quantity of methapyrilene per gram of dry resin to permit their use as medicinal carriers.

**Rate of Release Studies.**—Experiments were conducted to determine the rate of release of methapyrilene from cation exchange resins under uniform conditions approximating those of the upper gastrointestinal tract. The rate of release of the drug from the resin-adsorbate was determined by measuring the amount of methapyrilene released into 100 ml. of 0.1N hydrochloric acid at different time intervals. A sample of the resin-adsorbate equivalent to 100 mg. of methapyrilene hydrochloride was used. It was mixed with the acid in a sintered-glass-bottomed funnel with a capacity of approximately 190 ml. and the resin-adsorbate was kept well suspended by an electric stirrer, with its rotational speed controlled by a variac. At the end of fifteen minutes, the exchange medium was filtered with the aid of a water aspirator, and the filtrate was analyzed spectrophotometrically for the amount of methapyrilene which had been released into it. Fresh exchange solution was added to the resin adsorbate and the process was repeated for the desired number of exchange periods.

Experiments with a carboxylic acid resin-adsorbate indicated that this adsorbate had a very rapid rate of release of methapyrilene into 0.1 N hydrochloric acid and is largely converted to the hydrogen form of the resin within fifteen minutes. Since a slower and more prolonged release of the drug was desired, the sulfonic acid type resins appear to be the carriers of choice for methapyrilene.

A series of experiments was conducted to determine the effect of particle size and degree of cross-linkage on the rate of release of methapyrilene into 0.1 N hydrochloric acid from adsorbates prepared from sulfonic acid cation exchange resins. The method described previously was used except that after four hours (16 fifteen-minute exchange periods), the time intervals were extended to one hour periods, and the process was continued for a total of twelve hours. The longer periods were necessary after it was found that beyond four hours the amount of methapyrilene released in a fifteen-minute period was less than 1%. The data from these experiments are presented in Figs. 2-4.

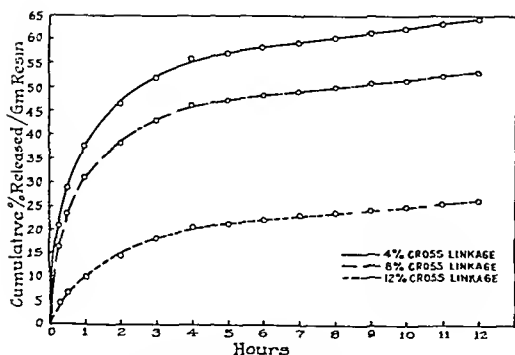


Fig 2—The effect of cross linkage on the rate of release of methapyrilene from Dowex 50, 50-100 mesh into 0.1 N HCl

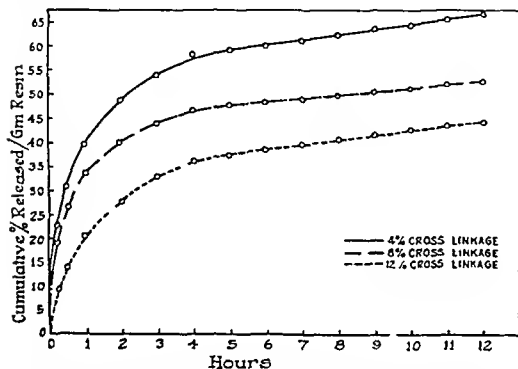


Fig 3—The effect of cross linkage on the rate of release of methapyrilene from Dowex 50, 100-200 mesh into 0.1 N HCl

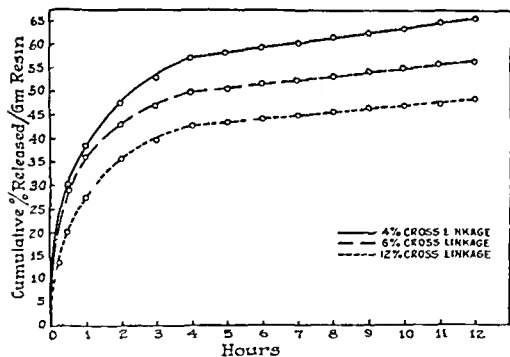


Fig 4—The effect of cross linkage on the rate of release of methapyrilene from Dowex 50, 200-400 mesh into 0.1 N HCl

Examination of these data shows that the rate of release of the drug was inversely related to the degree of cross linkage of the resins. Also, it reveals that there was a greater difference in the rate of release between 12 and 8% cross linked resins than between 8 and 4% cross linked resins. This indicates a critical resin porosity between 8 and 12% cross linkage, above which the rate of exchange is greatly reduced. Also, it appears that, with a decrease in particle size, the effect of cross linkage becomes relatively less.

The effect of particle size on the rate of release was less evident with resins of cross linkage below

8%. However, with the 8 and 12% cross linked resins, it was apparent that the rate of release was inversely related to the particle size.

Consideration of the rate of release, the extent of release over a twelve hour period, the palatability and suspendability of the various resins led to the selection of a resin with 4% cross linkage and a 200-400 mesh range for formulation and *in vivo* studies.

**Formulation Studies.**—Several nonionic suspending agents were used individually and in combinations in an attempt to formulate a stable and palatable suspension of the selected resin adsorbate. These experiments resulted in the following formula:

Resin adsorbate	q s
Vanillin, 5% in alcohol	1%
Span 85 <sup>3</sup>	0.2%
Methylcellulose 1,500 cps 1% solution	
Simple syrup	aa q s 100%

This formulation resulted in an excellent product with no noticeable sedimentation after standing for thirty days. The product was palatable except for a slight grittiness, which is inherent in the resin particles. Span 85 was included in this product to minimize foaming which occurred when it was agitated.

**In Vivo Studies.**—The *in vivo* studies involved a comparison of the protection against histamine induced asthma in the intact guinea pig provided by a solution of methapyrilene hydrochloride and a suspension of the resin-adsorbate administered orally. The method employed was a modification of the one used by Lee, *et al* (15). It is based on the principle that inhalation of a histamine aerosol by guinea pigs induces bronchial asthma, which can be lessened in severity by an antihistaminic agent.

Guinea pigs weighing from 500-800 Gm were used. They were fasted for twelve hours prior to each experiment. The histamine aerosol was produced by nebulizing with a DeVilbiss No. 40 atomizer a 1:80 solution of histamine diphosphate with seventy mm of Hg air pressure. The time required for each untreated animal to develop prominent asthmatic symptoms, as manifested by laborious breathing and a prominent gasp, was recorded as the control time in seconds with an electric timer. Control tests were conducted with each animal before each experiment, and animals with a control time much greater than two minutes were not used. The animals were permitted to rest one hour after the control tests, then were dosed with the desired compound by stomach intubation.

The individual animals were tested hourly, with only two exceptions, by exposing them to the histamine aerosol. The exceptions occurred during one experiment with animals number 3 and 9 which were tested at one half hour intervals after eight hours. The criterion for measurable protection was arbitrarily selected as an exposure of twice the control time before the appearance of prominent asthmatic symptoms. This criterion corresponds to the one used by Fainberg, *et al* (16). This method allowed a direct comparison

<sup>3</sup> Span 85 is a trade name of the Atlas Powder Co. for their brand of sorbitan trioleate.

of the protection provided by orally administered methapyrilene and the resin-adsorbed methapyrilene.

Four experiments were conducted with six guinea pigs. Three animals were given the resin-adsorbate and three were given a solution of methapyrilene hydrochloride. The animals were crossed over at two-day intervals so that all animals received both the resin adsorbate and the methapyrilene twice.

Table II shows the results of these experiments.

TABLE II.—PROTECTION AGAINST HISTAMINE AEROSOL DYSPNEA IN GUINEA PIGS BY METHAPYRILENE AND RESIN-ADSORBED METHAPYRILENE

Methapyrilene Controls		Resin-Adsorbed Methapyrilene	
Animal No.	Hours	Animal No.	Hours
2	5	1	4
4	6	3	6
6	7	9	6
1	5	2	8
3	8	4	4
9	4	6	5
2	5	1	..
4	3	3	9.5
6	5	9	8.5
1	..	2	8
3	6	4	7
9	7	6	6
Average	5.5	..	6.5

The animals dosed with the resin-adsorbed drug had an average duration of protection of six and one-half hours, whereas the control animals had a duration of protection of five and one-half hours. The difference, however, was not significant as indicated by the Fisher "t" test ( $t_{20} = 1.46$ ;  $0.2 > p > 0.1$ ). The value of 1.46 for "t" indicated a probability level less than 0.2, but greater than 0.1. Although the results seemed to indicate a longer duration of protection with the resin-adsorbed drug, the difference was not statistically significant in light of the large variation in the animal responses. The experiments demonstrated only that the resin-adsorbed drug possessed pharmacological activity at least equal, if not superior, to the solution of methapyrilene hydrochloride.

## DISCUSSION

The *in vitro* studies indicated that while the initial release of methapyrilene from its resin-adsorbate is relatively rapid, the rate decreases with time. The *in vivo* studies indicated that this initial release of drug is sufficiently rapid to provide a therapeutic effect in the guinea pig within thirty minutes or less.

Also, the *in vitro* experiments showed that without the addition of fresh exchange medium, the exchange process soon reached apparent equilibrium. When this condition is obtained in a biological system, it is to be expected that the rate of release of the drug will depend essentially on its rate of absorption through the intestinal villi. After a certain point in the process, the rate of absorption through the intestinal villi probably will exceed the rate of release of the drug from its adsorbate, at which time the blood level of the drug may begin to fall.

Since only a portion of the drug is released during the early stage of the process, the blood level should not exceed that necessary for therapeutic effects by such a great margin as that which occurs when all of a drug is immediately available for absorption. Thus, undesirable side effects may be diminished or eliminated. Another advantage of the use of ion exchange resins for medicinal carriers is the elimination of the variations in the blood levels of drugs which often occur with repeated doses of conventional dosage forms. This statement is based on the fact that the rate of release of the drug from its resin-adsorbate is continuous over a long period of time and decreases uniformly.

Further work is indicated to prove conclusively the above hypothesis. The use of a drug tagged with a radioactive isotope seems suited for such work because this technique permits the direct measurement of the drug in the body fluids and tissues.

## SUMMARY

1. The relative capacity of several cation exchange resins to adsorb methapyrilene was determined.

2. The rate of release of methapyrilene from its resin-adsorbates was studied *in vitro*. The carboxylic acid resin-adsorbate exhibited a very high exchange rate in an acid medium (0.1 N HCl). The sulfonic acid cation exchange resins are applicable for a controlled, sustained release of ionic drugs, and the rate of release may be controlled by the proper selection of the cross-linkage and particle size of the resin.

3. A stable suspension of a selected resin-adsorbate was formulated without materially affecting the rate of release of methapyrilene.

4. Methapyrilene adsorbed on a sulfonic acid cation exchange resin possessed pharmacological activity at least equal, if not superior, to a solution of methapyrilene hydrochloride, as measured by the protection against histamine-induced asthma in the intact guinea pig.

## REFERENCES

- (1) Adams, B. A., and Holmes, E. L., *Soc. Chem. Ind. London*, 54, 1(1935).
- (2) Kunin, R., "Ion Exchange Resins," 2nd ed., John Wiley & Sons, Inc., New York, N. Y., 1958, p. 9.
- (3) *Ibid.*, p. 292.
- (4) *Ibid.*, p. 293.
- (5) Segal, H. L., Miller, L., and Morton, J., *Proc. Soc. Exptl. Biol. Med.*, 74, 218(1950).
- (6) Ikai, K., *J. Invest. Dermatol.*, 23, 411(1954).
- (7) Nashed, W., and Sperandio, G. J., *Drug Standards*, 23, 100(1955).
- (8) Fiedler, W. C., and Sperandio, G. J., *THIS JOURNAL*, 46, 44(1957).
- (9) *Ibid.*, pp. 47-51.
- (10) Neuhauser, I., *Arch. Internal Med.*, 93, 58(1954).
- (11) Gustus, E. L., U.S. pat. 2,697,059, Dec. 14, 1954.
- (12) Chaudhry, N. C., and Saunders, L., *J. Pharm. and Pharmacol.*, 8, 957(1956).
- (13) Freed, S. C., Hays, E. E., and Keating, J. W., *Ann. Internal Med.*, 44, 1136(1956).
- (14) Martin, E. W., and Harrison, J. W. E., *THIS JOURNAL*, 39, 390(1950).
- (15) Lee, A. M., Dinwiddie, W. G., and Chen, K. K., *J. Pharmacol. Exptl. Therap.*, 90, 83(1947).
- (16) Feinberg, S. M., Malkiel, S., Berstein, T. B., and Hargis, B. J., *ibid.*, 99, 195(1950).

# The Influence of Some Suspending Agents on the Release of a Soluble Medicament from Solution\*

By G. D. REDMAN, J. E. CHRISTIAN, and GLEN J. SPERANDIO

A method for comparing the influence of suspending media on the dialysis of a soluble compound is described. Different suspending agents affected the rate at which the iodide ion dialyzes from solution, indicating that the nature of the agent is a variable which influences the release of the compound. It is demonstrated that the use of selected suspending agents makes possible the modification of release rates of certain soluble drugs from solution.

IN RECENT YEARS a number of new approaches to the preparation of dosage forms for oral administration have been made which are intended to produce a sustained level of drug action. Most of the work thus far has been in the development of tableted or encapsulated medicinal substances, although liquid preparations intended for oral administration which would have the ability to release incorporated active ingredients over an extended period of time may find application in the areas of geriatric, pediatric, and psychiatric medicine. Therefore, this project was initiated in an attempt to find a way to prepare liquid medication with sustained release properties. It was thought that one possible way of influencing drug release from solutions might be to add certain substances which might delay the process of dialysis; and some commonly used suspending agents were chosen for initial investigation.

A number of workers have utilized radioisotopes to study the release of substances from pharmaceutical preparations. Johnston, *et al.* (1), studied the absorption of  $\text{Na}^{24}\text{Cl}$  from the ointments by the intact skin of rats. Cyr, *et al.* (2), measured the absorption of  $\text{NaI}^{131}$  which had been incorporated into ointment bases through the skin of rats. Lux, *et al.* (3), studied the iodide ion permeability of living frog membranes using sodium radioiodide.

An *in vitro* procedure for the determination of the release of anions and cations from emulsified ointment bases was reported by Stark, *et al.* (4). Trace quantities of  $\text{NaI}^{131}$  and of  $\text{Hg}^{203}(\text{NO}_3)_2$  were incorporated independently into emulsified ointment bases and the dialysis of the ions in a dialysis apparatus especially adapted for the detection of radioactivity present in the dialysate was used.

Other workers have employed dialysis procedures to study the dispersion of a wide variety of substances (5-10).

This investigation utilized iodine<sup>131</sup>-labeled iodide ion to study the effect of different suspending agents on the dialysis of the iodide ion through a semipermeable membrane.<sup>1</sup> The effect of different concentrations of two selected suspending agents on dialysis was also determined.

## EXPERIMENTAL

The composition and suppliers of the individual suspending agents which were chosen for study are shown in Table I.

TABLE I.—COMPOSITION AND SOURCE OF SELECTED SUSPENDING AGENTS

Suspending Agent	Composition	Supplier
Attagel 20	Activated attapulgitic clay	Minerals & Chemical Corp. of America, Menlo Park, N. J.
Cato 8	Cationic corn starch derivative	National Starch Products Inc., Plainfield, N. J.
Jaguar gum	Galaetomannan from endosperm of guar seed	Stein Hall & Co., Inc., New York, N. Y.
Locust bean gum	Galaetomannan from endosperm of locust bean seed	Stein Hall & Co., Inc., New York, N. Y.
Methocel 400	Methylcellulose U. S. P., a methyl ether of cellulose	Dow Chemical Co., Midland, Mich.
Pectin N. F.	Complex hydrophilic colloidal carbohydrate, chiefly partially methoxylated polygalacturonic acids	S. B. Penick & Co., New York, N. Y.
Ramalin G	Amylopectin	Stein Hall & Co., Inc., New York, N. Y.
Superlose	Amylose	Stein Hall & Co., Inc., New York, N. Y.
Tragacanth U. S. P.	Complex hydrophilic carbohydrate composed of glucuronic acid, arabinose, and methoxylated acids	S. B. Penick & Co., New York, N. Y.
Veegum	Magnesium aluminum silicate	R. T. Vanderbilt Co., New York, N. Y.

\* Received April 8, 1959, from Purdue University, School of Pharmacy, Lafayette, Ind.  
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August, 1959.

<sup>1</sup> Type SS seamless, regenerated cellulose dialysis tubing, average pore radius 21 Å. Visking Corp., Chicago, Ill.

One per cent dispersions of each suspending agent were prepared in 200-ml. quantities. Two grams of the agent were dispersed in approximately 100 ml. of U. S. P. phosphate buffer solution (pH 7.0) to form a slurry. To the slurry 4 ml. of a 0.5% solution of potassium iodide U. S. P. and 0.2 Gm. of methylparaben U. S. P. were added, and the volume was brought to 200 ml. with additional buffer solution. The slurry was quantitatively transferred to a Liquidizer<sup>2</sup> and mixed at low speed. A 500- $\lambda$  quantity of solution containing approximately 4  $\mu$ c. of iodine<sup>131</sup> was added to a 35-ml. aliquot of the suspending media. The added activity imparted approximately 3,000 counts per minute per 5 ml. of the sample as determined by the counting procedure described below.

A dialysis apparatus described by Stark (4) was modified for use in this study. A glass cylinder used as the sample holder was firmly secured to the remainder of the dialysis apparatus by stretching rubber bands between glass projections fused to the apparatus. A rubber washer, cut from  $\frac{3}{4}$  inch stock, was placed around the base of the sample holder and positioned flush with its ground-glass surface to prevent the glass from cutting the membrane. A thin film of silicone lubricant was evenly spread on the ground-glass surface of the sample holder to reduce the possibility of sample leakage.

The membrane was attached to the dialysis apparatus by the procedure described by Stark (4) and normal saline solution was used as the dialysate. The apparatus was placed in a constant temperature bath to maintain the temperature of the dialysate at  $37^\circ \pm 1^\circ$ .

Five-milliliter samples, accurately measured and checked by weighing, of the suspending media prepared for test were placed in the sample holder of the dialysis apparatus. The dialysate was circulated through a closed system past the surface of the semipermeable membrane and around a counting tube to detect the migrating ions. A gamma-sensitive bismuth cathode counting tube was used in conjunction with a Model 1620 analytical count rate meter.<sup>3</sup> The per cent probable error of a single reading after equilibrium was reached, based on a fifty-second time constant and an expected count, was 0.85. An Esterline-Angus Model AW milliammeter graphic recorder<sup>4</sup> was used to provide a continuous recording of the dialysis of the iodide ion.

Prior to each determination a 5-ml. quantity of the radiolabeled suspending media, accurately determined and equal to that used in the release studies, was placed in the lumen of the counting chamber and dispersed in the dialysate. The activity of this sample corrected for background represented 100% activity of the sample and was used as the standard to express the percentage release of the iodide ion.

## RESULTS

The influence of equal concentrations of suspending agents on the dialysis of the iodide ion is shown in Fig. 1. Each curve represents the average of

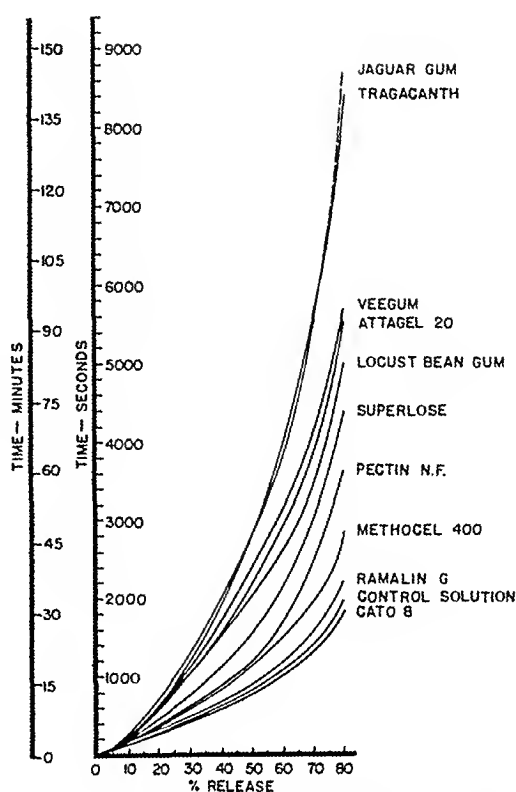


Fig. 1.—The influence of 1% concentrations of various suspending agents on the dialysis of the iodide ion.

five determinations of the dialysis of the iodide ion. For control purposes the dialysis of the iodide ion exclusive of the influence of the suspending agent was determined. The data show that the release of the iodide ion is not linear with respect to time. All of the release patterns are asymptotic in character and approach the asymptote at approximately 80% release of the available iodide ion.

If the period of time required to reach a given level of iodide dialysis from a suspending medium exceeded by 10% that which was required for the dialysis of an equal percentage of iodide ion from the control solution, the suspending agent was considered to have a significant influence on the release of the iodide ion from solution.

A test for homogeneity of variances among the means of the variable, time, was made at the 25% and also at the 75% release levels, and included all of the media and the control (11). In both instances it was established that the variances are heterogeneous. Therefore, the hypothesis that the interval of time required to obtain a given percentage of release of the iodide ion from the control solution and from a suspending medium are equal was tested using a *t* test (variances not equal) (12). The use of the *t* test was restricted to instances in which there was a reasonable doubt that a significant difference between the control and the media existed.

Only one of the media gave evidence of a capacity to increase the rate of passage of the iodide ion across the membrane. This agent, Cato 8, was shown by the application of a two-tailed *t*

<sup>2</sup> Knapp Monarch Co., St. Louis, Mo.

<sup>3</sup> Nuclear-Chicago Corp., Chicago, Ill.

<sup>4</sup> Esterline-Angus Co., Inc., Indianapolis, Ind.



test at an alpha level of 0.05 to effect a significant increase in the speed of the dialysis over that of the control. The reduction in the time required to release a specific amount of iodide ion was not significantly different from that of the control until 50% of the available iodide ion had dialyzed from the sample. The control time was approximately three minutes longer than that required to obtain the release of 80% of the available iodide ion from the media containing Cato 8.

The plot of the dialysis of the iodide ion from a 1% dispersion of Ramalin G shown in Fig. 1 indicates that the dialysis was retarded. The *t* test was used to test the means at the 25, 50, 75, and 80% release levels to determine if a significant difference exists between the means. A significant difference was found to exist at all levels tested. To obtain the dialysis of 80% of the iodide ion from the sample took about thirty-five minutes, for an equal dialysis from the control solution it took approximately thirty-three minutes.

All of the other media tested also retarded the dialysis of the iodide ion. Jaguar gum and tragacanth produced the greatest delay in the release followed by Veegum and Attagel 20, locust bean gum, Superlose, pectin N F, Methocel 400, and Ramalin G in the order named.

The effect of concentration of the suspending agent on the dialysis rate of the iodide ion was also investigated. In Fig. 2 are shown the relative influences of 0, 0.5, 1.0, and 1.5% concentrations of Methocel 400 and Jaguar gum on the dialysis of the iodide ion. The data show that at the concentrations tested the agents retard the dialysis of the iodide ion, but that the dialysis rate is not pro-

portional to the concentration of the agent in the media. Media containing 0.5 and 1.0% Methocel 400 released 80% of the contained iodide ion in approximately 2,850 seconds (forty-eight minutes), and that a 1.5% concentration of the agent took seven minutes longer. The control time was 1,977 seconds (thirty-three minutes).

Media containing 0.5% Jaguar gum released 80% of the available iodide ion in approximately 3,310 seconds (fifty-five minutes). The media containing 1.0 and 1.5% Jaguar gum showed no significant differences and released 80% of the iodide ion in 8,486 seconds (one hundred and forty minutes).

## CONCLUSIONS

1 The procedure used in this investigation has proved to be a satisfactory method for comparing the influence of suspending media on the dialysis of a soluble compound.

2 Different suspending agents were shown to affect the rate at which the iodide ion dialyzes from solution. The release rates indicate that the nature of the agent is a variable affecting the release of the compound.

3 Equal concentrations of the suspending agents tested affect the dialysis of the iodide ion in the following manner: Cato 8 increased the rate over that of the control; all others retarded the dialysis of the iodide ion. Jaguar gum and tragacanth produced the greatest delay followed by Veegum and Attagel 20; locust bean gum, Superlose, pectin N F, Methocel 400, and Ramalin G, in the order named. Attagel 20 and Veegum affected iodide ion release to the same degree. Tragacanth U S P and Jaguar gum also showed similar effects.

4 An increase in concentration of Methocel 400 and Jaguar gum does not alter the dialysis rate in proportion to the change in concentration.

5 The use of selected suspending agents enables the modification of release rates of certain soluble drugs from solution.

## REFERENCES

- (1) Johnston, G. W., and Lee, C. O., *THIS JOURNAL*, 32, 78 (1943).
- (2) Cyr, G. N., Skann, D. M., Christian, J. F., and Lee, C. O., *ibid.*, 38, 615 (1949).
- (3) Lux, R. L., and Christian, J. E., *Am. J. Physiol.*, 162, 193 (1950).
- (4) Stark, J. F., Christian, J. F., and DeKay, H. G., *THIS JOURNAL*, 47, 223 (1958).
- (5) Hober, R., "Physical Chemistry of Cells and Tissues," Blakiston Co., Philadelphia, Pa., 1945, p. 15.
- (6) Higuchi, T., Kuramoto, R., Kennon, L., Flanagan, T. L., and Plok, A., *THIS JOURNAL*, 43, 616 (1951).
- (7) Berthier, R. W., and Meyer, L. M., *Proc. Soc. Exptl. Biol. Med.*, 94, 169 (1957).
- (8) Klotz, I. M., Walker, F. M., and Pivan, R. B., *J. Am. Chem. Soc.*, 68, 1168 (1946).
- (9) Cavallito, C. J., and Jewell, R., *THIS JOURNAL*, 47, 167 (1958).
- (10) Cavallito, C. J., and O'Dell, T. B., *ibid.*, 47, 169 (1958).
- (11) Dixon, W. J., and Massey, F. J., Jr., "Introduction to Statistical Analysis," McGraw-Hill Co., Inc., New York, N. Y., 1957, p. 179.
- (12) *Ibid.*, p. 23.

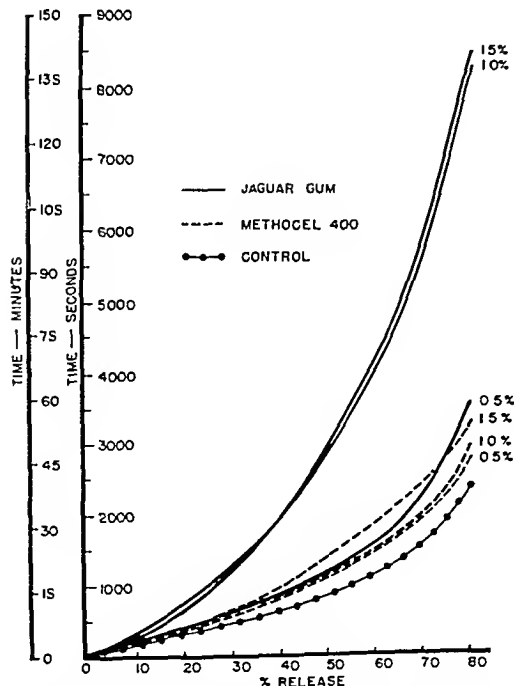


Fig. 2—The influence of varying concentrations of two different suspending agents on the dialysis of the iodide ion.

# Effect of Hydration on Hydrocholeresis in Rats\*

By SISTER DANIEL JOSEPH, S. T. COKER, and L. L. EISENBRANDT

Hydration produces a significant increase in normal bile flow and a significant decrease in specific gravity. The hydrocholeretic response to sodium dehydrocholate is significantly greater in hydrated rats than in non-hydrated controls. Restimulation of non-hydrated animals does not increase bile flow above the peak of initial stimulation. Hydration does not appear to alter the mechanism of action of sodium dehydrocholate, since the onset and duration of action is the same in both control and hydrated animals. Only a potentiation of hydrocholeresis is evident.

**M**ECHANISMS involved in bile formation have been studied by many investigators. Tanturi and Ivy (1) studied the effect of vascular changes in bile formation. Berman, *et al.* (2), noted that unconjugated, oxidized bile acids exert a greater osmotic effect, which results in excretion of a more hydrous bile than the conjugated acids. Cook, *et al.* (3), noted that dehydrocholic acid increased the excretory rate and biliary clearance of water, sodium, and chloride. They concluded that the mechanism of action of hydrocholeretic agents primarily involves stimulation of a filtration mechanism.

These observations as well as work in our laboratory led to the study of the effect of hydration on the hydrocholeretic response in rats.

## EXPERIMENTAL

Twenty-five Sprague-Dawley albino rats weighing between 235-345 Gm. and maintained under standard laboratory conditions were divided into three groups. Anesthesia was initiated with ether and then replaced by thiopental sodium. The bile duct and trachea were cannulated. For the hydrated animals 0.1% thiopental sodium was dissolved in i. v. infusion fluid, and for nonhydrated animals 1% aqueous solution was given i. v. as needed. Hydration was effected by constant i. v. infusion of 2% of body weight with 0.9% sodium chloride over a period of one hour (4, 5). The infusion apparatus consisted of a Phipps and Bird syringe driver adapter with an electric kymograph. A 30-cc. syringe was used and the kymograph was operated at first speed; 0.079 cc. of fluid was administered per minute. Hydrocholeresis was produced by i. v. administration of 200 mg./Kg. of sodium dehydrocholate. Since it was obse

sodium dehydroch  
minute period, all  
at five minute ir

each treatment. Bile flow was reported in mg. wet weight/five-minute interval. (Specific gravity of bile

was determined by weighing in a tared syringe. Tests for significance were made using the Student t test and analysis of variance.

## RESULTS AND DISCUSSION

A thirty-minute period of control or normal bile flow was determined in each of 25 animals prior to stimulation with sodium dehydrocholate. Group I consisted of 10 animals. After the control period (A-B, Fig. 1), they were subdivided into two groups; six which were stimulated only (B-D, Fig. 1) and four which were hydrated (F-G) and then stimulated (G-H). Group II consisted of 10 animals and differed from group I in that each animal was stimulated, allowed to return to normal, then hydrated for one hour and restimulated to obtain the hydrated-stimulated flow (A-H, Fig. 1). To determine whether the increase in hydrocholeresis after hydration and restimulation might be due to residual sodium dehydrocholate, a second dose of sodium dehydrocholate was administered to 5 nonhydrated animals in group III as soon as the original bile flow returned to normal (D-E, Fig. 1). No significant increase in bile flow was noted in these nonhydrated, restimulated animals. Summarized data on the effect of hydration on bile flow and specific gravity are in Table I.

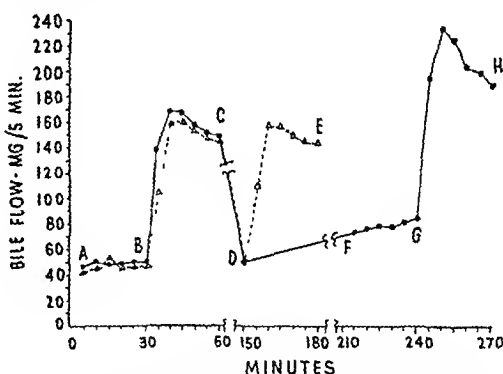


Fig. 1.—Mean Hydrocholeresis in 25 hydrated (—○—) and nonhydrated (—△—) rats. Flow areas A-B, normal; B-C, nonhydrated stimulated; C-D return to normal; D-E, nonhydrated restimulated; D-F, hydration priming; F-G hydrated; G-H, hydrated restimulated.

In every case there was a significant increase in bile flow and a decrease in specific gravity after one hour of hydration. Also, hydrocholeresis after stimulation was significantly greater than in non-hydrated animals. The onset and duration of the hydrocholeretic response was the same after hydration and stimulation, indicating that the effect produced by hydration is quantitative and not qualitative, with no change in the mechanism of action of the hydrocholeretic agent.

Spencer (6) considers that the primary event of bile formation might be the active transfer of bile

\* Received August 21, 1959, from the University of Kansas City, School of Pharmacy, Kansas City, Mo.  
Presented to the Scientific Section, A. Ph. A. Cincinnati meeting, August 1959.

TABLE I—EFFECT OF HYDRATION ON HYDROCHOLERESIS IN RATS

	Mean Bile Flow, mg /30 min	P <sub>a</sub> value	Increase from Normal, %	Mean Specific Gravity	P Value	Decrease from Normal %
Group I <sup>b</sup> (10 rats)						
Normal	48.9 ± 4.8 <sup>c</sup>			1.129		
Hydrated (4 rats)	80.5 ± 6.8	<0.01	65	1.118	<0.001	13
Stimulated (6 rats)	139.0 ± 12.5		166	1.087		37
Hydrated-stimulated	208.1 ± 11.8	<0.01	326	1.067	<0.001	53
Group II (10 rats)						
Normal	49.4 ± 2.6			1.129		
Stimulated	145.2 ± 15.6	<0.001	218	1.082	<0.001	42
Hydrated	80.2 ± 4.6		62	1.106		20
Hydrated-stimulated	212.9 ± 21.0	<0.05	331	1.065	<0.001	57
Group III (5 rats)						
Normal	46.7 ± 3.1			1.126		
Stimulated	145.2 ± 9.8		211	1.095		28
Nonhydrated-restimulation	146.5 ± 11.4	Not sig	215	1.087	Not sig	34

<sup>a</sup> Determined by analysis of variance. t test used for specific gravity.

<sup>b</sup> Group I, 10 rats, subdivided after normal period into four for hydration and hydrated stimulation, 6 for stimulation only.

<sup>c</sup> Standard error of mean.

acids from hepatic cells into bile capillaries. In the case of hydrated animals, a potentiation of hydrocholerisis could result from increased availability of water which readily passes into the bile capillaries in the presence of the osmotically-active sodium dehydrocholate. Brauer (7) feels that the mechanism of bile formation involves not only the active transport of bile acids, but also water, sodium, potassium, chloride, and glucose from blood to bile.

## REFERENCES

- (1) Tanturi, C. A., and Ivy, A. C., *Am. J. Physiol.*, 121, 61 (1938).
- (2) Berman, A. L., Snapp, E., Ivy, A. C., Atkinson, A. J., and Hough, V. S., *Am. J. Digest. Diseases*, 7, 333 (1940).
- (3) Cook, D. L., Lawler, C. A., and Green, D. M., *J. Pharmacol. Exptl. Therap.*, 110, 293 (1954).
- (4) Wesson, L. G., and Anslow, W. B., *Am. J. Physiol.*, 162, 677 (1950).
- (5) Wolf, A. V., *ibid.*, 143, 572 (1945).
- (6) Sperber, I., *Pharmacol. Revs.*, 11, 128 (1959).
- (7) Brauer, R. W., *J. Am. Med. Assoc.*, 169, 1462 (1959).

## A Gum from Rain Lily, *Cooperia pedunculata*\*

By WALLACE L. GUESS†, NATHAN A. HALL, and L. WAIT RISING

A heretofore undescribed plant principle was extracted from the bulbs of *Cooperia pedunculata*; the principle was purified and characterized as a new gum with several physical properties similar to those of other pharmaceutical gums. A preliminary chemical investigation of the gum from *C. pedunculata* (commonly known as rain lily) revealed that the gum gave reactions common to many of the known gums. It was also found that the gum was a polysaccharide and the principal sugar was identified as mannose by chromatographic procedures and the melting point of its phenylhydrazone. The infrared spectrum of a dried film of the gum was grossly similar to the spectra of most other gums commonly used in pharmacy, yet specific enough that a rapid identification of the gum would be possible. Rain lily gum exhibited rheological properties usually associated with natural hydrophilic colloids. The effect of aging, temperature, and pH on the viscosity of rain lily gum was also investigated.

GUMS are of major importance as pharmaceutical agents and they enjoy a large number of applications in other fields as well. For some gums, the United States is wholly dependent upon

foreign sources; the annual importation of acacia and tragacanth has been reported as ten to fourteen million pounds (1, 2). The bulb of the rain lily, *Cooperia pedunculata*, which is native to the state of Texas, contains a highly viscous mucilaginous principle of potential pharmaceutical usefulness. The work reported here consists of the isolation and partial purification of this mucilaginous material, a potential domestic source of gum, and a preliminary investigation of its chemical nature.

\* Received August 21, 1959, from the University of Washington, College of Pharmacy, Seattle.

Abstracted in part from a thesis presented to the Graduate School, University of Washington by Wallace L. Guess, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Fellow of the American Foundation for Pharmaceutical Education. Present address: College of Pharmacy, University of Texas, Austin.

Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.

## EXPERIMENTAL

Since the growing season of the rain lily plant is short and uncertain, large quantities of the bulbs were dug in the early Spring during the flowering and seed stages and stored in a freezing cabinet at  $-10^{\circ}$ . The freshly dug bulbs were washed and the tops broken off, leaving a stem about 5-6 cm long attached to the bulb. These bulbs were sealed in large plastic bags, frozen, and kept frozen until ready to be used. Little or no obvious decomposition of the bulbs occurred within a one year storage period in the cold, however, it was found that if the bulbs were left at room temperature or rapidly dried in warm air, there was decomposition and a considerable loss of gum.

**Isolation and Partial Purification of the Gum.**—The outer coat was peeled from the frozen bulbs and 100 Gm to 150 Gm portions were chopped in a Waring Blendor with 500 ml of purified water until homogeneous. Each portion was then diluted to approximately 750 ml with water and placed in a stoppered Erlenmeyer flask. The flasks were placed in a refrigerator and allowed to stand for two days with occasional shaking. The liquid extract was removed from the pulp material by straining through gauze. The residue from each flask was again extracted by maceration with fresh water for two days and strained. The combined aqueous extracts were strained through muslin to remove the small amount of pulp which had passed through the gauze.

Microscopic examination of the aqueous extract showed the presence of starch granules and crystals of calcium oxalate. To remove the starch and most of the calcium oxalate, four 200 ml centrifuge tubes were filled with the extract and centrifuged in an International centrifuge, Size 2, model V, at 2500 r p m for fifteen minutes. This procedure was found to remove all of the starch granules (as determined by the iodine test) and most of the calcium oxalate, the remainder of which was removed during further purification.

The starch-free crude aqueous extract was poured into large, glass, flat bottomed evaporating dishes ( $10 \times 6 \times 2$  inches), frozen, and dried for approximately thirty hours in a Stokes freeze drying apparatus (model 2004LX3). The supporting trays for the dishes of frozen material were left at room temperature, while the lower, condensing trays were cooled by a refrigeration unit to  $-30^{\circ}$  to  $-40^{\circ}$ , and the pressure inside the chamber was reduced to approximately 1 mm. After about twelve hours the trays supporting the dishes of material were heated to  $40^{\circ}$  by circulating warm water and maintained at this temperature until the material completely dried. As the water evaporated from the dishes, the pressure inside the chamber dropped to a constant level of about 0.5 mm. The total time required for drying the extracts was about thirty hours. The product remaining in the dishes was a whitish buff colored, light, flaky material that could be easily reduced to a fine powder by trituration. The average yield of dried, crude rain lily gum was about 10% of the total weight of the fresh frozen bulb weight.

Since the crude material was known to contain some calcium oxalate and possibly some soluble organic material, 20-Gm portions of dried crude

material were resuspended in 200 ml of hot water, and this suspension was poured in a fine stream into one liter of warm 95% alcohol, with vigorous stirring. A white, rosy precipitate of mucilaginous material was collected, resuspended in hot water, and reprecipitated in warm alcohol. The precipitate was then washed with 95% alcohol and dried in the Stokes freeze drying apparatus as described previously. The average yield of purified rain lily gum from this procedure was about 25% of the total weight of the dried crude extract. Approximately three pounds of the purified rain lily gum was prepared, powdered in a ball mill, mixed thoroughly, and stored in a dark, cool place.

**Chemical Examination of Rain Lily Gum.**—One of the standard characteristics of acacia, tragacanth (3), and gums in general is precipitation from dispersions by lead subacetate. When 1 ml of a 5% solution of lead subacetate was added to 5 ml of a 4% dispersion of rain lily gum, heavy, white precipitate formed. To ascertain the carbohydrate nature of the material, a sample was tested for reducing sugars with Benedict's reagent. The reaction was negative, but on acidifying with hydrochloric acid, boiling the solution to hydrolyze the gum, and alkalizing with sodium hydroxide, a positive Benedict's test was obtained. These tests indicated that part of the gum material under study was composed of a polysaccharide.

Partridge (4) was the first to report a separation of sugars by the use of paper chromatography. Since this worker's original report, numerous authors (5) have used paper chromatography to separate and identify simple sugars, sugar derivatives, and hydrolytic products of gums (6). To apply the paper chromatographic methods to rain lily gum, a hydrolysate was prepared by treating 1 Gm of the gum with 1 N sulfuric acid (20 ml) at  $95^{\circ}$  for ten hours. The excess sulfuric acid was neutralized with barium carbonate and the neutral solution filtered.

A sheet of Whatman No 1 chromatography paper ( $29 \text{ cm} \times 46 \text{ cm}$ ) was prepared by drawing a pencil line 5 cm from the bottom of the paper, and 0.1 ml each of the hydrolysate and several sugar solutions were spotted along this line at 3 cm intervals. The known sugar solutions (1 mg in 1 ml water) were galactose, mannose, rhamnose, and mixtures of these three sugars. The hexoses chosen as comparison sugars in this experiment were three sugars that are common to many of the known gums. Pentoses were not used as reference sugars since preliminary chromatographic work had shown that the  $R_f$  value of known samples was not near the  $R_f$  value for any sugar spots obtained from the rain lily hydrolysate.

The chromatographic procedure was carried out by the ascending method with two solvent systems: a mixture of *n* butanol, glacial acetic acid, and distilled water (4:1:5) or ethyl acetate, distilled water, and pyridine (2:2:1). The bottom of a chromatographic jar was covered with the aqueous phase and the chamber sealed for twenty-four hours to ensure atmospheric saturation. One end of the prepared chromatography paper containing the sugar spots was then dipped in a Petri dish containing the mobile, nonaqueous phase of the above-mentioned solvents, the jar sealed, and the chromatograms allowed to develop for eighteen hours at  $25^{\circ}$ .

The paper was removed from the chromatographic jar and the solvent front marked. After being dried in the air, the paper was sprayed with 0.1 *N* aniline in glacial acetic acid and then heated in an oven at 100° for three minutes, resulting in distinct pink or brown spots from the reducing sugars (7). The distance the spots had moved from the starting line was measured and the  $R_f$  values calculated.

A comparison of the  $R_f$  values obtained (see Table I) with mannose and one of the sugars from the hydrolysate of rain lily gum showed that the values were nearly the same, whether the developing solvent was acidic or basic. These close values indicated that the hydrolysate of rain lily gum contained mannose. The second spot shown in the hydrolysate had an  $R_f$  value higher than any of the known sugars used, therefore no tentative identification of this spot was made. It was felt that the spot did not represent a uronic acid because  $R_f$  values reported (8) for several uronic acids were lower than the  $R_f$  values for the corresponding sugars while the  $R_f$  value for this unknown spot was higher than any of the test sugars.

Further evidence for mannose was provided by a phenylhydrazine derivative which showed an unchanged melting point when mixed with authentic mannose phenylhydrazine.

TABLE I.—A COMPARISON OF THE  $R_f$  VALUES OF CERTAIN KNOWN SUGARS WITH RAIN LILY HYDROLYSATE

Sugar	$R_f$ Values	
	n-Butanol, 40% Glacial HAc, 10% Water, 50%	Ethyl Acetate, 40% Water, 40% Pyridine, 20%
1. Galactose	0.203	0.582
2. Mannose	0.225	0.650
3. Rhamnose	0.367	0.736
4. Rain Lily	Spot a 0.223 Spot b 0.754	Spot a 0.643 Spot b 0.915
Mixture of 1, 2, 3	1 0.199	1 0.579
	2 0.224	2 0.646
	3 0.363	3 0.721

**Infrared Spectrum.**—Newburger and co-workers (9, 10) have reported a method for identifying naturally-occurring gums by the use of infrared analysis. According to their method, a film of rain lily gum for examination was cast on a smooth spatula, coated with Desicote.<sup>1</sup> The spatula was dipped into a 4% dispersion of rain lily gum and dried over a steam bath. A piece of film large enough for analysis was removed with a razor blade, and this film was dried for six hours at 100°. The film was placed in a KBr cell which was positioned in a Baird-Atomic infrared spectrophotometer (model 4-55) and the infrared spectrum recorded.

Examination of the infrared spectrum (Fig. 1) reveals that its gross characteristics are very similar to that of acacia, tragacanth, guar, karaya, and many other gums; however, there are enough distinctive features in its spectrum that an identification of rain lily gum should be possible.

**Viscosity Characteristics of Rain Lily Gum.**—Although the reproducibility of rheological measurements made on dispersions of gums is generally poor

<sup>1</sup> Desicote is a silicone preparation purchased from Beckman Instruments, Inc., Pasadena, Calif.

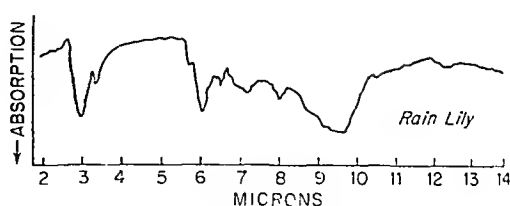


Fig. 1.—Infrared spectrum of rain lily gum.

due to numerous factors, it was felt that some useful information could be obtained by examining some of the flow characteristics of the rain lily gum dispersion. Measurements were made with a Brookfield viscometer, model LVF, with spindle No. 4, at  $25^\circ \pm 1^\circ$ . A 4% (w/v) dispersion of rain lily gum was prepared by placing the freeze-dried material in purified water, allowing it to hydrate in a refrigerator for forty-eight hours, and stirring with a mechanical stirrer. Dispersions of acacia (40%, w/v) and tragacanth (4%, w/v) were prepared similarly for comparison. In all measurements, after the samples had attained temperature equilibrium, the spindle was allowed to complete five revolutions before readings were taken and the values recorded represent the average of at least three readings. To assess the constancy of measurements, Brookfield readings at 30 r. p. m. were converted to "centipoises" by means of the Newtonian calibration chart supplied with the instrument. For several 4% rain lily gum dispersions prepared on different days, the average viscosity was 7,900 c. p. s. (standard deviation  $\pm 494$ ).

Figure 2 illustrates the flow curves (up-curve only) of the rain lily gum dispersion compared to acacia and tragacanth. Rain lily gum dispersions show the typical pseudo-viscous or pseudo-plastic characteristics which are common to many plant gums. As expected, the viscosity of the acacia dispersion was too low for valid measurement within the experimental limitations.

**Preliminary Pharmaceutical Evaluation.**—Rain

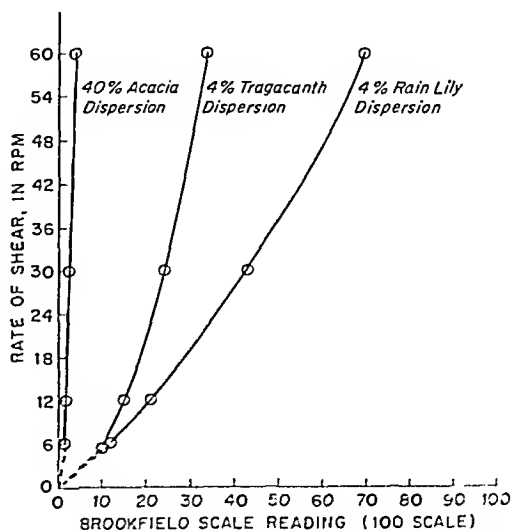


Fig. 2.—Flow curves for some gums. Brookfield viscometer, No. 4 spindle.

lily gum was used to prepare 50% oil in water emulsions of liquid petrolatum and cod liver oil to examine its emulsifying ability. In this study rain lily gum (2.5%), tragacanth (2.5%), and acacia (25%) liquid emulsions were investigated over a two-month period by mean globule diameter method of Smith and Grinling (11). As a primary emulsifier, rain lily gum was comparable to tragacanth and inferior to acacia; thus it probably should be classified as an auxiliary emulsifier.

A number of suspension and semisolid cream formulas in which gums have been used were prepared with the rain lily gum. The preparations were generally satisfactory and further studies of the pharmaceutical applications of the gum are being made.

### SUMMARY

A new gum from the bulbs of *Cooperia pedunculata* has been isolated and partially purified. A preliminary chemical investigation revealed that the gum was a polysaccharide containing mannose as a component monosaccharide. An in-

frared spectrum of the gum was similar to other gums and appeared to be characteristic enough for identification. The viscosity characteristics indicated that the gum was similar to tragacanth. A survey of possible pharmaceutical uses indicated that the gum could be classed as an auxiliary emulsifier and that further study of its pharmaceutical properties should be done.

### REFERENCES

- (1) Mason, C. F., *Chem. Ind. London*, 53, 680(1943).
- (2) Mantel, C. L., "The Water Soluble Gums," Reinhold Publishing Corp., New York, N. Y., 1947, pp. 19, 64.
- (3) Scott, W. W., "Standard Methods of Chemical Analysis," 5th Ed., D. Van Nostrand Co., Inc., New York, N. Y., 1939, p. 1675.
- (4) Partridge, S. M., *Nature*, 158, 270(1946).
- (5) Lederer, E., and Lederer, M., "Chromatography," Elsevier Publishing Co., New York, N. Y., 1953.
- (6) Oiseth, von D., *Pharm. Acta Helv.*, 29, 251(1954).
- (7) Hough, L., Jones, J. K. N., and Wadman, W. H., *J. Chem. Soc.*, 1950, 1702.
- (8) Lederer, E., *loc. cit.*, p. 161.
- (9) Newburger, S. H., Jones, J. H., and Clark, G. R., *Proc. Sci. Sect., Toilet Goods Assoc.*, No. 18, (1952).
- (10) Newburger, S. H., Jones, J. H., and Clark, G. R., *ibid.*, No. 19, 25(1953).
- (11) Smith, L., and Grinling, G. N., *Quart. J. Pharm. and Pharmacol.*, 3, 354(1930).

## An Investigation of the Effect of Ultrasonic Waves on the Rates of Hydrolysis of Procaine and Butethamine Hydrochlorides\*

By G. D. FENN and P. F. BELCASTRO

Data are presented showing the effect of ultrasonic waves on the rates of hydrolysis of procaine and butethamine hydrochlorides. Under the experimental conditions employed, significant changes in the rates were not observed with the insonated samples.

A NUMBER of investigators (1-4) have reported that ultrasonic vibrations initiate or accelerate chemical reactions. Faust (5) investigated the chemical effects of ultrasound on glutathione and other compounds. Robert (6) discusses the oxidation of aromatic nuclei under the influence of ultrasonic vibrations and Prudhomme and Grabar (7) report depolymerization as a result of ultrasonic irradiation. Many such effects have been attributed to a local heating effect (2, 8, 9). This heating effect has been estimated to be as high as several hundred degrees and has been attributed to adiabatic compression of cavitation bubbles (10). In view of these investigations, the possibility of accelerating the rate of certain

chemical reactions applied to pharmaceuticals was realized. This communication reports the effect of ultrasonic waves at a frequency of 400 kc. per second on the rates of hydrolysis of procaine and butethamine hydrochlorides, by comparison with noninsonated temperature accelerated hydrolysis. These comparative hydrolysis rates were investigated at several different hydrogen ion concentrations. It was hoped that the effects would be of sufficient magnitude to be of value in accelerated stability testing of pharmaceutical solutions.

### EXPERIMENTAL

**Calibration of the Generator.**—The ultrasonic generator employed in this study was constructed at Purdue University and was designed for use with a barium titanate transducer. The generator is rated at 250 watts with a variable frequency range. A Hypersonic transducer, model BU-301, with a focused bowl of barium titanate was employed. The generator was calibrated by determining the wattage produced at various rheostat settings by multiplying the power amplifier voltage by the power amplifier current, as shown by appropriate meters on the generator. To compensate for various trans-

\* Received August 21, 1959 from the School of Pharmacy, Purdue University, West Lafayette, Ind.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Available under trade name Monocaine hydrochloride, Novocel Chemical Mfg. Co.

mission losses, 65% of this calculated wattage was estimated to be the true energy reaching the sample (11). All insonation experiments were conducted at a frequency of 400 kc. per second and an energy level of approximately 125 watts at the focal point of the transducer.

**Hydrolysis of Procaine Hydrochloride.**—Twenty-five-milliliter samples of 0.1% procaine hydrochloride solution with various hydrogen ion concentrations were hydrolyzed under the influence of ultrasonic vibrations. The results obtained were compared with those for 100-ml. samples of 0.1% procaine hydrochloride samples hydrolyzed under the influence of temperature at 25.0° and at 30.0° ± 0.01°. Temperature control in the case of the noninsonated samples was maintained by use of a Sargent thermometer controlled bath, model S-84810. The cooling coil of the transducer was not effective in maintaining precise temperature control of the insonated samples. The temperatures reported for the insonated samples represent the arithmetic mean of the temperatures observed at the time of sampling for analysis. The sample solutions were prepared by diluting a 1.0% solution of procaine hydrochloride to 0.1%, using an appropriate buffer solution to which 0.2% sodium bisulfite was added as an antioxidant. The pH of the resulting samples was determined before and after hydrolysis, using a Beckman model G pH meter. The composition of the buffers employed is shown by Table I.

TABLE I.—COMPOSITION OF MODIFIED SØRENSEN BUFFERS

Ingredient	Approximate pH				
	8	9	10	11	12
NaHSO <sub>3</sub> , Gm./L.	2.00	2.00	2.00	2.00	2.00
H <sub>3</sub> BO <sub>3</sub> , Gm./L.	6.98	10.85	7.34	6.11	5.41
NaOH, Gm./L.	3.05	4.30	4.80	4.80	4.80
0.1 M HCl, ml./L.	437.50	125.00	0.00	0.00	0.00

The analytical procedure for the determination of residual procaine hydrochloride was essentially that of Higuchi (12), modified as follows: A 1-ml. portion of the sample was diluted to 100 ml. with a pH 9.5 buffer composed of 96.131 Gm./L. of H<sub>3</sub>BO<sub>3</sub> and 4.000 Gm./L. of NaOH. The absorbance of the resulting solution was then determined at wavelengths of 287 mμ and 271.5 mμ using a Beckman model DU spectrophotometer. The per cent residual ester was then calculated according to the following formula as reported by Higuchi (12):

$$\% \text{ residual ester} = 100 \times \frac{(k - k_{\text{PABA}})}{(k_{\text{Proc}} - k_{\text{PABA}})}$$

where  $k$  = the observed absorbance of the partially hydrolyzed sample diluted to 0.001% with respect to original procaine hydrochloride;  $k_{\text{PABA}}$  = the absorbance of *p*-aminobenzoate ion equivalent to 0.001% concentration of procaine hydrochloride; and  $k_{\text{Proc}}$  = the absorbance of 0.001% procaine hydrochloride, all readings being taken at a fixed wavelength and pH 9.5.

The absorbances determined at 271.5 mμ served

as an internal check on any side reactions and limited the useful data taking time since the molar extinction coefficients for procaine hydrochloride and PABA are the same at this wavelength. Figure 1 shows the first-order hydrolysis observed at pH 9.9 under the various conditions employed.

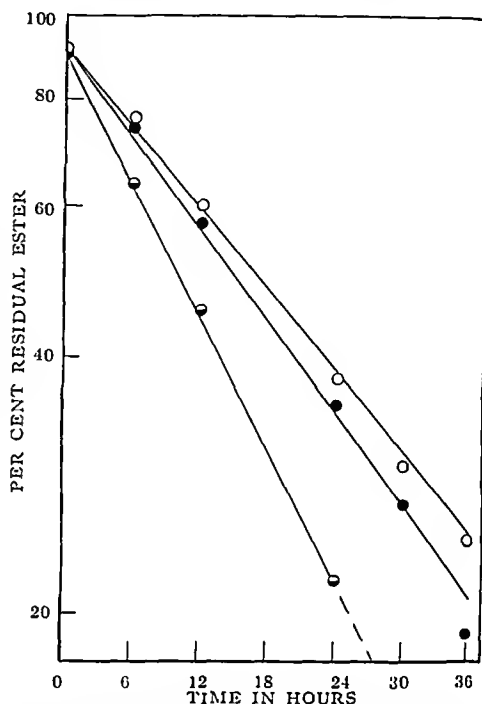


Fig. 1.—Hydrolysis of procaine hydrochloride in solution at pH 9.9; O, noninsonated at 25.0°; ○, noninsonated at 30.0°; ●, insonated at 26.7°.

**Hydrolysis of Butethamine Hydrochloride.**—The procedure followed for butethamine hydrochloride was similar to that used for procaine hydrochloride. No solutions of butethamine hydrochloride were hydrolyzed at pH 11 or 12 since preliminary experimentation indicated that the half lives would be less than fifteen minutes. The formula for calculation of per cent residual procaine ester was modified as follows for use with butethamine hydrochloride.

$$\% \text{ residual ester} = 100 \times \frac{(k - k_{\text{PABA}})}{(k_{\text{Bute}} - k_{\text{PABA}})}$$

where  $k$  = the observed absorbance of the partially hydrolyzed sample diluted to 0.001% with respect to original butethamine hydrochloride;  $k_{\text{PABA}}$  = the absorbance of *p*-aminobenzoate ion equivalent to 0.001% concentration of butethamine hydrochloride; and  $k_{\text{Bute}}$  = the absorbance of 0.001% butethamine hydrochloride, all readings being taken at a fixed wavelength and pH of 9.5.

It was determined that the wavelength of maximum absorption for butethamine hydrochloride is 287 mμ and the isosbestic point with PABA occurs at 266 mμ. Figure 2 shows the first-order hydrolysis observed at pH 7.9 under the various conditions employed.

**Comparison of Half Lives.**—In order to show the comparative rates of hydrolysis of the various samples under all the experimental conditions employed





# A Preliminary Study of the Alkaloidal Principles of *Ceanothus Americanus* and *Ceanothus velutinus*\*

By CHARLES W. ROSCOE† and NATHAN A. HALL

The presence of a complex mixture of alkaloids in the root barks of *Ceanothus Americanus* and *Ceanothus velutinus* has been demonstrated by means of circular paper partition chromatography. Preliminary fractionation studies have yielded one crystalline alkaloid, m. p. 251–252° (K), from *Ceanothus velutinus*. The alkaloid was partially characterized by its infrared spectrum. The total alkaloid fraction from *Ceanothus Americanus* and the ether-soluble and ether-insoluble alkaloid fractions from *Ceanothus velutinus* were tested for hypotensive activity and found to exhibit insignificant activity at the reported dosage levels. Since previous investigators have reported on the hypotensive activity shown by various alkaloidal extracts from *Ceanothus Americanus* it is recommended that the fractionation studies be continued in the hope of isolating sufficient quantities of individual alkaloids for further pharmacological testing and chemical characterization.

THE RESULTS of early chemical investigations of *Ceanothus Americanus* (1–4) suggested the presence of a complex mixture of alkaloids in the root bark of this shrub. The only reported attempt to resolve the alkaloid mixture by chromatographic methods was unsuccessful (5). In the light of these results it seemed that a re-examination of the alkaloidal material should be undertaken.

Other species of *Ceanothus* have received little or no attention from investigators. The species *Ceanothus velutinus* grows so prolifically in easily accessible areas in the state of Washington as to warrant more than casual interest. Richards and Lynn (6), in 1934, submitted evidence for the presence of alkaloidal material in this species but no subsequent investigations have been reported.

The primary objective of this study was that of attempting to demonstrate the homogeneous or heterogeneous nature of the alkaloidal principles in the two plant species by means of improved chromatographic techniques. It was also hoped that some pure alkaloids might be isolated for use in future chemical characterization and biological activity studies. Previous reports on the hypotensive activity shown by alkaloidal extracts obtained from *Ceanothus Americanus* (5, 7, 8) served to lend additional impetus to the study.

## EXPERIMENTAL

### Source of Plant Material

The roots of *Ceanothus velutinus* were collected in the immediate vicinity of U. S. Highway 2, approxi-

mately 15 miles east of the summit of Stevens Pass in the state of Washington. The bark was removed from the freshly dug roots and allowed to dry in a prior to comminution in a Wiley mill fitted with the smallest size screen. The material was collected at various times during the summer months. No attempt was made to ascertain the optimum harvest time. Herbarium samples of *Ceanothus velutinus* were authenticated by Dr. C. L. Hitchcock, Department of Botany, University of Washington.

The root bark from *Ceanothus Americanus* was supplied by Flint, Eaton & Co., Decatur, Ill.

### Isolation of Alkaloid Fractions

Preliminary studies indicated that the initial extraction of the alkaloids from either plant species was best accomplished with methanol or ethanol. No prior alkalization was necessary. Defatting of the drug was found to be desirable since appreciable quantities of vegetable fat or wax were present.

The general scheme for obtaining the alkaloid fractions is outlined in Fig. 1. Amorphous alkaloid fractions were isolated from the root bark of *C. velutinus* and *C. Americanus* in yields of 0.20% and 0.16%, respectively. Specific rotations were found to be as follows: *C. velutinus*,  $[\alpha]_D^{25} = +205^\circ$  (CHCl<sub>3</sub>); *C. Americanus*,  $[\alpha]_D^{25} = +150^\circ$  (CHCl<sub>3</sub>).

The acid fractions obtained from the ether extracts (Fig. 1) are currently under investigation.

### Resolution of Alkaloid Mixtures by Circular Paper Partition Chromatography

**Method.**—Manian (5) screened an extensive number of solvent systems in an unsuccessful attempt to resolve the alkaloidal mixture in *C. Americanus* by means of paper-strip chromatography. Likewise, we investigated a number of solvent systems and techniques without success. Finally, some degree of resolution was achieved with the ascending, moist, buffered paper technique which Levin and Fischbach (9) employed for the resolution of a mixture of veratrum alkaloids with *n*-butyl acetate-*n*-butanol solvent systems containing acetic or formic acid. However, the resulting chromatograms exhibited elongated, diffuse spots with considerable tailing which made it difficult to locate individual spots with any degree of certainty. By utilizing the basic features of this method and incorporating modifications of the circular paper

\* Received August 31, 1959, from the School of Pharmacy, University of Washington, Seattle.

† Fellow, American Foundation for Pharmaceutical Education, 1955–1958. E. Mead Johnson Memorial Fellow, 1957–1958. Present address: School of Pharmacy, Montana State University, Missoula.

This paper is abstracted in part from a dissertation presented to the Graduate School of the University of Washington by Charles W. Roscoe in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

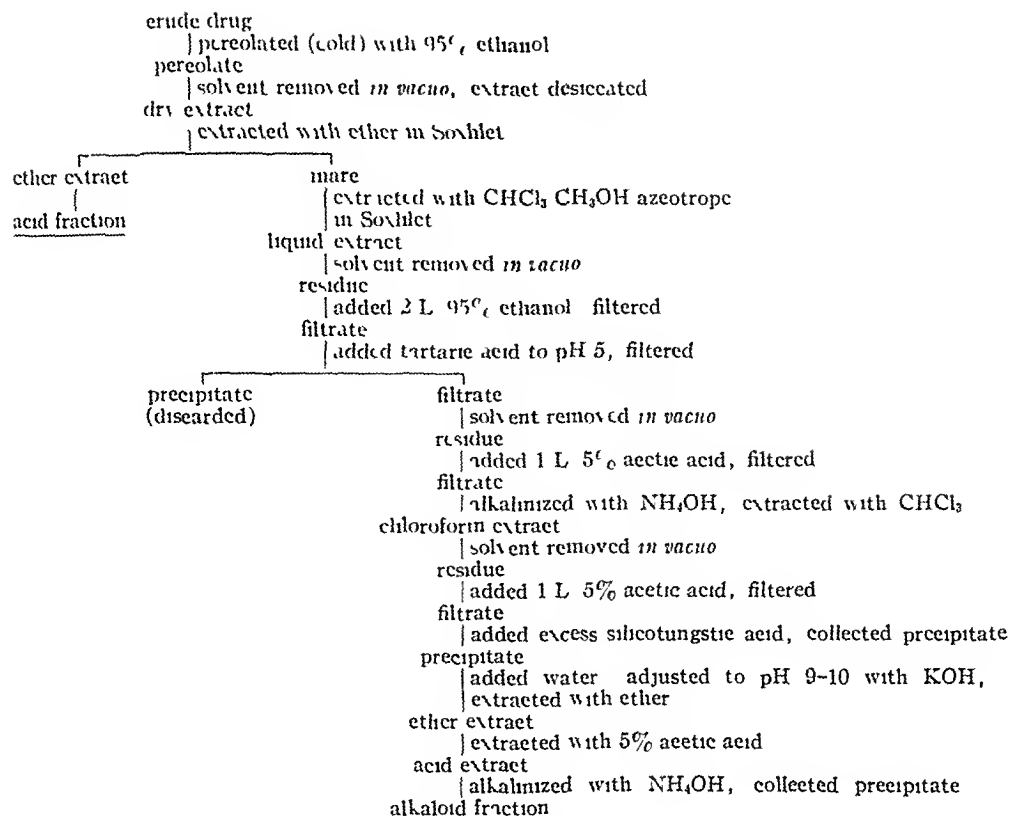


Fig 1—Scheme for isolation of alkaloid fractions

technique described by Rao (10), satisfactory resolution was eventually achieved. Optimum results were obtained using the resolving solvent system *n*-butyl acetate-*n*-butanol-acetic acid, 25:5:1 and 25:1:1 (by volume).

**Apparatus and Materials.**—The chromatography container used in these studies consisted simply of a glass casserole cover about 18 cm in diameter, which served as the reservoir for the developing solvent, and a heavy desiccator lid which served as the cover. The edges of the two parts were ground so that a tight seal resulted when they were fitted together.

The solvents, *n*-butyl acetate and *n*-butanol, of the mobile phase were purified by distillation through a Todd precise fractionation assembly. Reagent grade glacial acetic acid was used without further treatment. McIlvaine's phosphate citrate buffer (11), pH 3.5, was used as the stationary phase.

**Procedure.**—A circle 18 cm in diameter was drawn on a sheet of Whatman No. 1 filter paper. A wick was fashioned by cutting a flap 1 cm wide and 2 cm long from the edge of the circle toward its center. The flap was then folded downward, perpendicular to the sheet, and a strip of filter paper stapled to the lower end of the flap in order for the wick to extend into the developing solvent. An arc of 2 cm radius with the fold of the wick as its center was drawn toward the center of the paper. Solutions of the alkaloid mixtures were applied at two points, 1.4 cm apart, along the arc. With development of the chromatograms the components of the mixtures appeared to progress radially

along imaginary lines intersecting the starting points and the center of the arc. The most consistent  $R_f$  values were obtained by measuring distances along these lines.

The paper, prepared in the described manner, was quickly drawn through the aqueous phosphate-citrate buffer solution and the excess moisture removed by blotting between paper towels. Quantities of 200 to 400 mcg of the alkaloid mixtures in chloroformic solution were spotted on the moist paper along the pencil drawn arc. The paper was then exposed to the air for several more minutes and when it had attained the desired degree of wetness, as determined largely by the feel of the paper, it was sandwiched between the casserole cover and the desiccator lid with the wick dipping into the developing solvent. The solvent front was allowed to travel about 14 cm, after which the chromatogram was removed, dried, and sprayed with a 0.01% chloroformic solution of bromophenol blue. On humidifying the chromatogram with a flow of steam the alkaloids appeared as blue bands on a yellow background. A reproduction of a typical chromatogram produced in this manner is shown in Fig 2.

**Results.**—Using the resolving solvent system *n*-butyl acetate-*n*-butanol-acetic acid, 25:5:1, five bands were observed on chromatograms of the *C. americanus* mixture and six bands on chromatograms of the *C. velutinus* mixture. The results are summarized in Table I.

Using the resolving solvent system *n*-butyl acetate-*n*-butanol-acetic acid, 25:1:1, seven bands

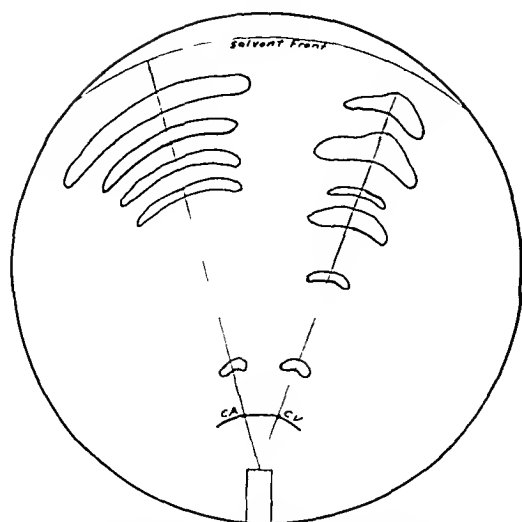


Fig 2—Reproduction of a typical alkaloid chromatogram developed with the solvent system *n*-butyl acetate : *n*-butanol : acetic acid (25 5 1, by volume). CA, *Ceanothus Americanus*, CV, *Ceanothus velutinus*

TABLE I— $R_f$  VALUES CALCULATED FROM CHROMATOGRAMS DEVELOPED WITH THE SOLVENT SYSTEM *n*-BUTYL ACETATE : *n*-BUTANOL : ACETIC ACID, 25 5 1 (BY VOLUME)

Band No.	<i>Ceanothus Americanus</i>		<i>Ceanothus velutinus</i>	
	Mean $R_f^a$	Average Error, $\pm\%$	Mean $R_f^a$	Average Error, $\pm\%$
1	0 17	4 7	0 18	5 6
2	0 64	3 1	0 42	1 4
3	0 72	2 8	0 60	0 7
4	0 81	2 5	0 66	1 5
5	0 92	1 1	0 80	1 0
6			0 92	2 2

<sup>a</sup> Average of five determinations

appeared on chromatograms of the *C. americanus* mixture and five bands on chromatograms of the *C. velutinus* mixture. The results are summarized in Table 11

#### Fractional Solution of Alkaloid Mixtures with Ether

In view of the apparent complexity of the alkaloid mixtures in both plant species it was hoped that a preliminary crude separation might serve to facilitate further fractionation by more elaborate methods

**Method.**—The mixture of alkaloids from *C. Americanus* was subjected to continuous extraction in a Soxhlet apparatus with anhydrous, peroxide-free ether for a total of five days. The extractive obtained after a forty-eight-hour extraction period was designated fraction 1; the extractive obtained after an additional seventy-two-hour extraction period was designated fraction 2. The remaining residue was designated fraction 3

The mixture of alkaloids from *C. velutinus* was separated into two ether fractions by a similar procedure. The ether extractive obtained after a 96-hour extraction period was designated fraction 1; the remaining residue, fraction 2.

TABLE II— $R_f$  VALUES CALCULATED FROM CHROMATOGRAMS DEVELOPED WITH THE SOLVENT SYSTEM *n*-BUTYL ACETATE : *n*-BUTANOL : ACETIC ACID, 25:1:1 (BY VOLUME)

Band No.	<i>Ceanothus Americanus</i>		<i>Ceanothus velutinus</i>	
	Mean $R_f^a$	Average Error, $\pm\%$	Mean $R_f^a$	Average Error, $\pm\%$
1	0 18 <sup>b</sup>	9 4	0 08	5 3
2	0 24 <sup>b</sup>	4 2	0 41	2 4
3	0 40	3 0	0 60	1 7
4	0 54	1 9	0 68	1 2
5	0 67	1 5	0 79	0 4
6	0 81	0 7	..	
7	0 93	0 6		

<sup>a</sup> Average of five determinations.

<sup>b</sup> Detected under ultraviolet light.

**Results.**—Fractions 1, 2, and 3 from *C. Americanus* represented approximately 61, 22, and 17%, respectively, of the total weight of the original mixture. Specific rotations taken in chloroform at  $26 \pm 2^\circ$  were found to be  $-178$ ,  $-167$ , and  $-100^\circ$  for fractions 1, 2, and 3, respectively. Paper chromatograms of fraction 1, developed with the solvent system *n*-butyl acetate : *n*-butanol : acetic acid, 25 1 1, showed the presence of seven bands corresponding closely to those listed in Table 11. An eighth band ( $R_f$  0 09), which had not been previously detected on chromatograms of the original mixture, was also found. Four bands appeared on chromatograms of fraction 2 and two bands on chromatograms of fraction 3

Fractions 1 and 2 from *C. velutinus* represented approximately 44 and 56%, respectively, of the weight of the original mixture. Specific rotations taken in chloroform at  $26 \pm 2^\circ$  were found to be  $-215$  and  $-225^\circ$  for fractions 1 and 2, respectively. With the above solvent system paper chromatograms of fraction 1 showed the presence of five bands corresponding closely to those listed in Table 11. Two additional bands ( $R_f$ 's 0 14 and 0 24) were detected by examining the chromatograms under ultraviolet light. Three (and possibly four) bands could be detected on chromatograms of fraction 2.

#### Preliminary Fractionation by Column Adsorption Chromatography

**Method.**—The general procedure outlined by Reichstein and Shoppee (12) for the fractional elution of steroids from columns of alumina with an eluotropic series of solvents was similarly employed in an attempt to effect a further separation of the alkaloids in each of the ether fractions. Woelm aluminum oxide<sup>1</sup> (nonalkaline, activity grade 1) was used as the adsorbent in these studies. The fractions obtained by this method were examined individually by means of their paper chromatograms and specific rotations. The initial results of these studies are briefly reported here. A more detailed report will be published at a later date when further studies have been completed

**Results.**—The specific rotations of the *C. Americanus* fractions progressively decreased from  $-222$  to  $-47^\circ$  in the order that they were eluted from the alumina column. One of the amorphous fractions, which appeared to contain a single component on the basis of its paper chromatogram, melted at

<sup>1</sup> Available from Research Specialties Co., 2095 Hopkins Street, Berkeley 7, Calif

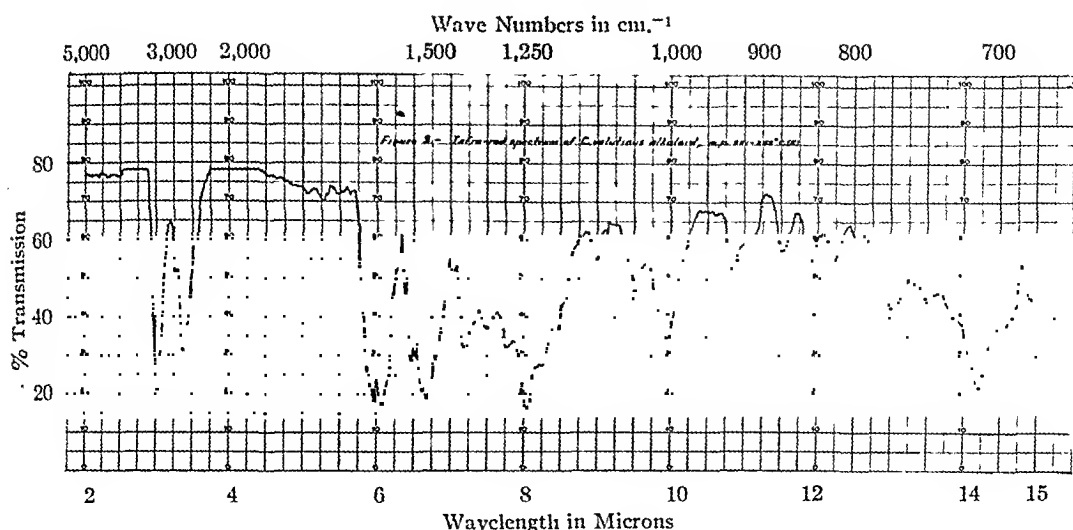


Fig. 3.—Infrared spectrum of *C. velutinus* alkaloid, m. p. 251-252° (K).

138-139° (K) but attempts to crystallize it were unsuccessful. All other fractions contained two or more major components.

The specific rotations of the *C. velutinus* fractions ranged from  $-292$  to  $-45^\circ$ . One of the crystalline fractions appeared to consist of one major component. The colorless, acicular crystals melted at  $251-252^\circ$  (K) after three recrystallizations from absolute ethanol. All other fractions contained two or more major components.

**Infrared Spectrum of *C. velutinus* Alkaloid.**—The infrared spectrum of the crystalline *C. velutinus* alkaloid, m. p.  $251-252^\circ$  (K), is shown in Fig. 3. The spectrum was taken on a solid sample in the form of a potassium bromide tablet using a Perkin-Elmer infrared spectrophotometer, model 21, equipped with a sodium chloride prism.

The single strong band at  $3,300\text{ cm}^{-1}$  falls within a frequency range which is usually assigned to the N—H stretching vibrations of secondary amines, secondary amides, and imines, although hydrogen-bonded OH groups may also absorb at this frequency. The band near  $2,900\text{ cm}^{-1}$  occurs at a frequency normally expected for C—H stretching vibrations.

The intense absorption at  $1,680\text{ cm}^{-1}$  suggests the presence of a conjugated carbonyl group. The equally intense absorption at  $1,640\text{ cm}^{-1}$  might be considered indicative of the carbonyl absorption of an amide group since all amides examined in the solid state have been found to absorb strongly near this frequency (13). The alternative possibility that the  $1,640\text{ cm}^{-1}$  band could arise from the C=C stretching vibrations of an  $\alpha\beta$  unsaturated keto group should also be considered. The weaker absorption near  $1,550\text{ cm}^{-1}$  might also be associated with an amide group (14).

Some indication for the presence of an aromatic structure is found in the absorption observed in the  $1,600-1,500\text{ cm}^{-1}$  region in conjunction with the sharp, relatively weak band which appears as a shoulder on the high frequency side of the  $2,900\text{ cm}^{-1}$  band (15). The band in the vicinity of  $1,390-1,380\text{ cm}^{-1}$  occurs in a region which has often been assigned to the C—CH<sub>3</sub> group.

The intense absorption at  $1,240\text{ cm}^{-1}$  falls within a frequency range normally assigned to the C—O

stretching vibrations of aryl and alkyl ethers as well as phenols, esters, acids, and other compounds possessing the  $\text{C}=\text{O}$  structure (16). The absorption near  $1,110\text{ cm}^{-1}$  could be due to an ether group.

The absorption bands near  $870$  and  $740\text{ cm}^{-1}$  in conjunction with those in the  $1,600-1,500\text{ cm}^{-1}$  region give some reason to suspect the presence of an aromatic heterocyclic ring system. The strong band near  $705\text{ cm}^{-1}$  could be due to out-of-plane  $\text{C}=\text{H}$  bending vibrations.

## PHARMACOLOGY

Although the literature contains several reports attesting to the hypotensive activity shown by various alkaloidal extracts derived from *C. Americanus*, no report on the pharmacological testing of *C. velutinus* extracts has been found. The results of a preliminary testing<sup>2</sup> of the ether-soluble and ether-insoluble alkaloid fractions of *C. velutinus* for hypotensive activity are reported here. A sample of the total alkaloids of *C. Americanus* was also tested. All tests were conducted on the anesthetized dog.

**Total Alkaloids of *C. Americanus* and Ether-Insoluble Alkaloids of *C. velutinus*.**—Intravenous doses below  $5\text{ mg./Kg.}$  produced no significant change in blood pressure. At dose levels of  $5$  and  $10\text{ mg./Kg.}$  a slight fall in blood pressure ( $15-20\text{ mm. Hg.}$ ) of brief duration (one to two minutes) was observed.

When administered intraduodenally at a dose level of  $10\text{ mg./Kg.}$  the ether-insoluble alkaloids of *C. velutinus* produced no change in blood pressure during a period of eighty-five minutes. The total alkaloids of *C. Americanus* caused a small increase in cardiac rate whereas the ether-insoluble fraction from *C. velutinus* produced no significant change. Neither of these substances interfered with adrenaline pressor responses nor did they show significant ganglionic blocking action.

**Ether-Soluble Alkaloids of *C. velutinus*.**—Intravenous doses of  $1, 5$ , and  $10\text{ mg./Kg.}$  evolved a fall in blood pressure ( $10-45\text{ mm. Hg.}$ ) of brief duration (one to eight minutes) accompanied by a

<sup>2</sup> The authors are indebted to the pharmacology staff of Mead Johnson & Co. for the pharmacological testing of the alkaloid samples for hypotensive activity.

small decrease in cardiac rate. This response resembles that observed after the i v administration of many types of drugs and was not considered to be significant. This fraction did not interfere with adrenaline pressor responses and exhibited no significant ganglionic blocking action.

### DISCUSSION

The results of the fractional solution with ether indicated that the majority of the alkaloids in each plant species resided in the more ether-soluble fractions. The appearance of previously undetected bands on paper chromatograms of the ether fractions suggested that there may be at least eight alkaloids in the root bark of *C. Americanus* and seven alkaloids in the root bark of *C. velutinus*, although the possibility that some of the bands might represent basic artifacts was considered.

The initial results of the fractionation by column chromatography indicated that this procedure might be employed, with certain refinements, for the final resolution of the alkaloid mixtures on a preparative scale. Unfortunately, the crystalline *C. velutinus* alkaloid, m. p. 251–252° (K), was isolated in extremely small yield and additional characterization studies were not possible due to the lack of sufficient material.

The insignificant hypotensive activity shown by

the sample of total alkaloids from *C. Americanus* is not in agreement with the findings of previous investigators. However, this may be due to the administration of lower dosages. Further testing at higher dosage levels was prevented by the lack of sufficient material. The possibility that the alkaloids might possess activity of a cumulative nature was considered.

### REFERENCES

- (1) Gordin, H. M., *Pharm. Rev.*, **18**, 266 (1900).
- (2) Clark, A. H., *Am. J. Pharm.*, **98**, 147 (1926).
- (3) Clark, A. H., *ibid.*, **100**, 210 (1928).
- (4) Bertho, A., and Liang, W. S., *Arch. Pharm.*, **271**, 273 (1933).
- (5) Manian, A. A., Ph.D. Thesis, Purdue University, 1954.
- (6) Richards, L. W. and Lynn, E. V., *THIS JOURNAL*, **23**, 332 (1934).
- (7) Groot, J. T. J., *Pharmacol. Exptl. Therap.*, **30**, 275 (1927).
- (8) Wastl, H., *Fed. Proc.*, **7**, 131 (1918).
- (9) Levine, J., and Fischbach, H., *THIS JOURNAL*, **44**, 543 (1955).
- (10) Rao, N. K. M., *Experientia*, **9**, 151 (1953).
- (11) Lange, N. A., "Handbook of Chemistry," 9th ed., Handbook Publishers, Inc., Sandusky, Ohio, 1956, pp. 932–953.
- (12) Reichstein, T., and Shoppee, C. W., *Discussions Faraday Soc.*, **7**, 305 (1949).
- (13) Bellamy, L. J., "The Infra-red Spectra of Complex Molecules," John Wiley & Sons, Inc., New York, N. Y., 1954, p. 180.
- (14) Bellamy, L. J., *ibid.*, p. 185.
- (15) Bellamy, L. J., *ibid.*, p. 59.
- (16) Bellamy, L. J., *ibid.*, p. 102.

## The Synthesis and Antifungal Evaluation of Certain Acetylenic Compounds\*

By JAMES A. WATERS† and GAIL A. WIESE

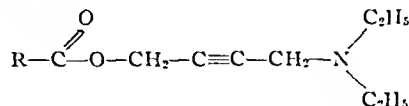
A number of 4-diethylamino-2-butyryl esters and 1,4-bis-N-substituted amino-2-butyne were prepared. The compounds, in the form of their hydrochloride and/or methiodide salts, were tested *in vitro* against three fungi causing dermatomycoses. Several compounds exhibited good activity in comparison to the control compound, sodium undecylenate. The most effective compound in this investigation was 4-diethylamino-2-butyryl *o*-toluate hydrochloride.

IN PAST YEARS, relatively few investigators have studied the antifungal activity of compounds containing an acetylenic component. The most recent paper in which a number of acetylenic compounds were evaluated was by Tanaka, *et al.* (1). The compounds were patterned after an acetylenic compound the same laboratory had previously isolated from the essential oil of *Artemisia capillaris*, which was reported to possess a high degree of antifungal activity (2).

It was of particular interest to note that in certain instances where acetylene carboxylic acids were studied for antifungal activity (3, 4), the free acids exhibited little fungistatic activity

but when esterified they were highly fungistatic.

In this investigation a number of acetylenic aminoesters were prepared, of the general formula shown below.



R = alkyl, alkenyl, or aromatic

Esters of the same pattern were of considerable interest to Marszak, *et al.* (5), and Biel, *et al.* (6), as potential pharmacodynamic agents. We were interested in studying the structure-antifungal activity relationships afforded by these three general types of esters.

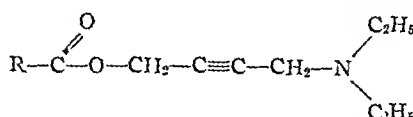
A few acetylenic diamines were also prepared. The free bases were converted into their hydrochloride and/or methiodide salts. The compounds prepared in this investigation are summarized in Tables I and II.

\* Received August 10, 1959 from the College of Pharmacy, State University of Iowa, Iowa City.

Abstracted from a dissertation submitted to the Graduate College of the State University of Iowa by James A. Waters in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August 1959.

† Fellow of the American Foundation for Pharmaceutical Education, 1958–1959. Present address: Department of Chemistry, University of Michigan, Ann Arbor.

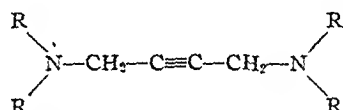
TABLE I—4-DIETHYLAMINO-2-BUTYNYL ESTERS



Ester	Yield, %	B p, °C <sup>a</sup>	Salt	No	Yield, %	M p, °C <sup>a</sup>	Formula	Analysis of N, %	
								Calcd	Found
Acetate <sup>a</sup>	63.0	85-89 <sup>m</sup>	HCl	1	54.6	93-94	C <sub>10</sub> H <sub>10</sub> NO <sub>2</sub> Cl <sup>b</sup>	6.38	6.25
Propionate	72.0	78-80							
n-Butyrate	75.4	78-81.5							
n-Hexanoate	81.0	94-95	HCl	2	76.2	62-63	C <sub>14</sub> H <sub>26</sub> NO <sub>2</sub> Cl <sup>c,h</sup>	5.08	5.18
n-Octanoate	89.9	115-117	CH <sub>3</sub> I	3	37.2	57-58.5	C <sub>17</sub> H <sub>32</sub> NO <sub>2</sub> I <sup>d</sup>	3.42	3.37
Crotonate	47.8	98-104	HCl	4	62.6	80-83	C <sub>12</sub> H <sub>20</sub> NO <sub>2</sub> Cl <sup>b</sup>	5.70	5.54
10 Undecenoate	70.9		CH <sub>3</sub> I	5	63.0	75-77	C <sub>20</sub> H <sub>36</sub> NO <sub>2</sub> I <sup>e</sup>	3.12	3.01
Sorbate	74.6	115-120	HCl	6	53.5	97-98	C <sub>14</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b</sup>	5.15	5.19
			CH <sub>3</sub> I	7	32.2	100-101	C <sub>16</sub> H <sub>24</sub> NO <sub>2</sub> I <sup>e</sup>	3.71	3.73
Benzoate <sup>a</sup>	65.3	125-126	HCl	8	75.0	98	C <sub>15</sub> H <sub>20</sub> NO <sub>2</sub> Cl <sup>b</sup>	4.96	4.80
			CH <sub>3</sub> I	9	44.4	91-92	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> I <sup>e</sup>	3.62	3.78 <sup>i</sup>
o-Toluate	73.6	121-121.5	HCl	10	82.4	135-136	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b</sup>	4.74	4.54
			CH <sub>3</sub> I	11	90.6	106-108	C <sub>17</sub> H <sub>24</sub> NO <sub>2</sub> I <sup>e</sup>	3.50	3.42
m-Toluate	81.8	124-126	HCl	12	27.6	160-162	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b,h,i</sup>	4.74	4.91
			CH <sub>3</sub> I	13	77.7	116-118.5	C <sub>17</sub> H <sub>24</sub> NO <sub>2</sub> I <sup>e</sup>	3.50	3.40
p-Toluate	64.3	133-134	HCl	14	93.0	113-114.5	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b</sup>	4.74	4.65
p-Nitrobenzoate	61.7	46-47 <sup>o</sup>	HCl	15	56.3	136-137	C <sub>16</sub> H <sub>19</sub> N <sub>2</sub> O <sub>4</sub> Cl <sup>b</sup>	8.57	8.25
			CH <sub>3</sub> I	16	66.6	134-136	C <sub>16</sub> H <sub>21</sub> N <sub>2</sub> O <sub>4</sub> I <sup>e</sup>	6.48	6.32

<sup>a</sup> Boiling points at 0.3-0.5 mm pressure, unless otherwise indicated. <sup>b</sup> Recrystallized from an absolute ethanol anhydrous ether mixture. <sup>c</sup> From absolute ethanol. <sup>d</sup> From absolute methanol anhydrous ether mixture. <sup>e</sup> No solvent was found satisfactory to recrystallize this compound. <sup>f</sup> A viscous oil which could not be distilled at 0.3-0.5 mm without decomposition. <sup>g</sup> Melting point. <sup>h</sup> Extremely hygroscopic. <sup>i</sup> Nitrogen analyses by a semimicro Kjeldahl method, unless otherwise indicated. <sup>j</sup> Analysis performed by Galbraith Laboratories, Knoxville, Tenn. <sup>k</sup> Melting points are uncorrected and were taken in capillary melting point tubes. <sup>l</sup> Melting point taken in a closed capillary tube. <sup>m</sup> B p at 1 mm pressure. <sup>n</sup> The free amine reported by Marszak, I, *et al*, *Compt rend*, 226, 1289(1948), b p 94° (0.5 mm). <sup>o</sup> *Ibid*, reported b p 163° (0.6 mm).

TABLE II—1,4-BIS-N-SUBSTITUTED AMINO-2-BUTYNES



R	Yield, %	B p, °C <sup>a</sup>	Salt	No	Yield, %	M p, °C <sup>b</sup>	Formula	Analysis of N, %	
								Calcd	Found
Diethylamino <sup>c</sup>	75.0	73-79	Di-HCl	17	63.5	204-205	C <sub>12</sub> H <sub>26</sub> N <sub>2</sub> Cl <sub>2</sub> <sup>e</sup>	10.40	10.06
			Di-CH <sub>3</sub> I	18	91.3	213.5-214	C <sub>14</sub> H <sub>30</sub> N <sub>2</sub> I <sub>2</sub> <sup>f</sup>	5.83	5.51
Pyrrolidino <sup>d</sup>	92.5	98-102	Di-HCl	19	74.8	222	C <sub>12</sub> H <sub>22</sub> N <sub>2</sub> Cl <sub>2</sub> <sup>e</sup>	10.56	10.49 <sup>i</sup>
			Di-CH <sub>3</sub> I	20	80.5	246	C <sub>14</sub> H <sub>26</sub> N <sub>2</sub> I <sub>2</sub> <sup>g,h</sup>	5.88	5.85 <sup>j</sup>

<sup>a</sup> Boiling points at 0.3-0.5 mm pressure. <sup>b</sup> All melting points with decomposition. <sup>c</sup> Reported b p 110° (10 mm), Wille, F., *et al*, *Ann*, 591, 177(1955). <sup>d</sup> Reported b p 93-95° (0.1 mm), Biel, J., and DiPierro, F., *J Am Chem Soc*, 80, 4609(1958). <sup>e</sup> Recrystallized from anhydrous ether-methanol mixture. <sup>f</sup> From absolute ethanol. <sup>g</sup> From absolute methanol. <sup>h</sup> Reported m p 239-240°, ref., see footnote d. <sup>i</sup> Analyzed by Galbraith Laboratories, Knoxville, Tenn.

## ANTIFUNGAL EVALUATION

The compounds were tested *in vitro* against *Trichophyton rubrum*, *Microsporum gypsum*, and *Microsporum audouinii*. The testing procedure was similar to that described by Goettseh (7), with the exceptions that aqueous solutions of the compounds and a seven-day incubation period were employed. At the end of the incubation period, the minimum zone of inhibition was measured. The zone of inhibition was the distance between the periphery of the paper disk and the colony growth. Compound 21, sodium undecylenate, was used as the control. The results of this antifungal study are given in Table III. Each value in this table represents an average of four zones of inhibition.

## DISCUSSION AND SUMMARY

The intermediates 4-chloro-2-butyne-1-ol and 1,4-dichloro-2-butyne were prepared by the procedure described by Bailey and Fujiwara (8). 4-Diethylamino-2-butyne-1-ol and the majority of the esters were prepared by the general procedures described by Biel, *et al* (6). The acetate and propionate esters were prepared by employing an excess of methyl acetate or methyl propionate without the use of the Dean-Stark water separator. The diamines were prepared by reacting an excess of the secondary amine with 1,4-dichloro-2-butyne.

The compounds deleted from Table III were inactive against all of the test organisms, which includes all of the 1,4-bis-N-substituted amino-2-butyne.

small decrease in cardiac rate. This response resembles that observed after the *in vivo* administration of many types of drugs and was not considered to be significant. This fraction did not interfere with adrenaline pressor responses and exhibited no significant ganglionic blocking action.

### DISCUSSION

The results of the fractional solution with ether indicated that the majority of the alkaloids in each plant species resided in the more ether-soluble fractions. The appearance of previously undetected bands on paper chromatograms of the ether fractions suggested that there may be at least eight alkaloids in the root bark of *C. Americanus* and seven alkaloids in the root bark of *C. velutinum*, although the possibility that some of the bands might represent basic artifacts was considered.

The initial results of the fractionation by column chromatography indicated that this procedure might be employed, with certain refinements, for the final resolution of the alkaloid mixtures on a preparative scale. Unfortunately, the crystalline *C. velutinum* alkaloid, m.p. 251–252° (K), was isolated in extremely small yield and additional characterization studies were not possible due to the lack of sufficient material.

The insignificant hypotensive activity shown by

the sample of total alkaloids from *C. Americanus* is not in agreement with the findings of previous investigators. However, this may be due to the administration of lower dosages. Further testing at higher dosage levels was prevented by the lack of sufficient material. The possibility that the alkaloids might possess activity of a cumulative nature was considered.

### REFERENCES

- (1) Gordin, H. M., *Pharm. Rev.*, **18**, 266 (1900).
- (2) Clark, A. H., *Am. J. Pharm.*, **98**, 117 (1926).
- (3) Clark, A. H., *ibid.*, **100**, 240 (1928).
- (4) Bertho, A., and Liang, W. S., *Arch. Pharm.*, **271**, 273 (1933).
- (5) Maman, A. A., Ph.D. Thesis, Purdue University, 1934.
- (6) Richards, L. W., and Lynn, E. V., *This Journal*, **23**, 332 (1934).
- (7) Groot, J. T. J., *Pharmacol. Exptl. Therap.*, **30**, 27 (1927).
- (8) Wastl, H., *Fed. Proc.*, **7**, 131 (1948).
- (9) Levine, J., and Fischbach, H., *This Journal*, **44**, 343 (1955).
- (10) Rao, N. K. M., *Experientia*, **9**, 151 (1953).
- (11) Lange, N. A., "Handbook of Chemistry," 9th ed., Handbook Publishers, Inc., Sandusky, Ohio, 1956, pp. 952–953.
- (12) Reichstein, T., and Shoppee, C. W., *Discussions Faraday Soc.*, **7**, 305 (1949).
- (13) Bellamy, L. J., "The Infra-red Spectra of Complex Molecules," John Wiley & Sons, Inc., New York, N. Y., 1951, p. 180.
- (14) Bellamy, L. J., *ibid.*, p. 185.
- (15) Bellamy, L. J., *ibid.*, p. 59.
- (16) Bellamy, L. J., *ibid.*, p. 102.

## The Synthesis and Antifungal Evaluation of Certain Acetylenic Compounds\*

By JAMES A. WATERS† and GAIL A. WIESE

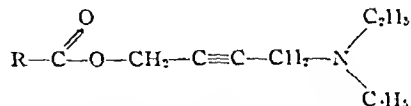
A number of 4-diethylamino-2-butyryl esters and 1,4-bis-*N*-substituted amino-2-butyrynes were prepared. The compounds, in the form of their hydrochloride and/or methiodide salts, were tested *in vitro* against three fungi causing dermatomycoses. Several compounds exhibited good activity in comparison to the control compound, sodium undecylenate. The most effective compound in this investigation was 4-diethylamino-2-butyryl *o*-toluate hydrochloride.

IN PAST YEARS, relatively few investigators have studied the antifungal activity of compounds containing an acetylenic component. The most recent paper in which a number of acetylenic compounds were evaluated was by Tanaka, *et al* (1). The compounds were patterned after an acetylenic compound the same laboratory had previously isolated from the essential oil of *Artemisia capillaris*, which was reported to possess a high degree of antifungal activity (2).

It was of particular interest to note that in certain instances where acetylene carboxylic acids were studied for antifungal activity (3, 4), the free acids exhibited little fungistatic activity

but when esterified they were highly fungistatic.

In this investigation a number of acetylenic aminoesters were prepared, of the general formula shown below:



R = alkyl, alkenyl, or aromatic

Esters of the same pattern were of considerable interest to Marszak *et al* (5), and Biel, *et al* (6), as potential pharmacodynamic agents. We were interested in studying the structure-antifungal activity relationships afforded by these three general types of esters.

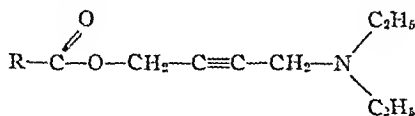
A few acetylenic diamines were also prepared. The free bases were converted into their hydrochloride and/or methiodide salts. The compounds prepared in this investigation are summarized in Tables I and II.

\* Received August 10, 1959 from the College of Pharmacy, State University of Iowa, Iowa City.

Abstracted from a dissertation submitted to the Graduate College of the State University of Iowa by James A. Waters in partial fulfillment of the requirements for the degree of Doctor of Philosophy, August 1959.

† Fellow of the American Foundation for Pharmaceutical Education, 1958–1959. Present address: Department of Chemistry, University of Michigan, Ann Arbor.

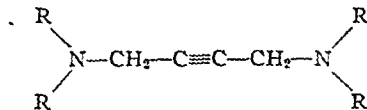
TABLE I.—1,4-DIETHYLAMINO-2-BUTYNYL ESTERS



Ester	Yield, %	B.p. <sup>a</sup> °C	Salt	No	Yield, %	M.p. <sup>a</sup> °C	Formula	Analysis of N, %	
								Calcd	Found
Acetate <sup>a</sup>	63 0	85-89 <sup>m</sup>	HCl	1	54 6	93-94	C <sub>10</sub> H <sub>10</sub> NO <sub>2</sub> Cl <sup>b</sup>	6 38	6 25
Propionate	72 0	78-80							
n-Butyrate	75 4	78-81 5							
n-Hexanoate	81 0	94-95	HCl	2	76 2	62-63	C <sub>14</sub> H <sub>26</sub> NO <sub>2</sub> Cl <sup>c,h</sup>	5 08	5 18
n-Octanoate	89 9	115-117	CH <sub>3</sub> I	3	37 2	57-58 5	C <sub>17</sub> H <sub>32</sub> NO <sub>2</sub> I <sup>d</sup>	3 42	3 37
Crotonate	47 8	98-104	HCl	4	62 6	80-83	C <sub>12</sub> H <sub>20</sub> NO <sub>2</sub> Cl <sup>b</sup>	5 70	5 54
10-Undecenoate	70.9	/	CH <sub>3</sub> I	5	63 0	75-77	C <sub>20</sub> H <sub>36</sub> NO <sub>2</sub> I <sup>e</sup>	3 12	3 01
Sorbate	74 6	115-120	HCl	6	53 5	97-98	C <sub>14</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b</sup>	5 15	5 19
			CH <sub>3</sub> I	7	32 2	100-101	C <sub>15</sub> H <sub>24</sub> NO <sub>2</sub> I <sup>e</sup>	3 71	3 73
Benzoate <sup>a</sup>	65.3	125-126	HCl	8	75 0	98	C <sub>15</sub> H <sub>20</sub> NO <sub>2</sub> Cl <sup>b</sup>	4 96	4 80
			CH <sub>3</sub> I	9	44 4	91-92	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> I <sup>e</sup>	3 62	3 78 <sup>i</sup>
o-Toluate	73 6	121-121 5	HCl	10	82 4	135-136	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b</sup>	4 74	4 54
			CH <sub>3</sub> I	11	90 6	106-108	C <sub>17</sub> H <sub>24</sub> NO <sub>2</sub> I <sup>e</sup>	3 50	3 42
m-Toluate	81 8	124-126	HCl	12	27 6	160-162	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b,h,i</sup>	4 74	4 91
			CH <sub>3</sub> I	13	77 7	116-118 5	C <sub>17</sub> H <sub>24</sub> NO <sub>2</sub> I <sup>e</sup>	3 50	3 40
p-Toluate	64 3	133-134	HCl	14	93 0	113-114 5	C <sub>16</sub> H <sub>22</sub> NO <sub>2</sub> Cl <sup>b</sup>	4 74	4 65
p-Nitrobenzoate	61 7	46-47 <sup>a</sup>	HCl	15	56 3	136-137	C <sub>15</sub> H <sub>19</sub> N <sub>2</sub> O <sub>4</sub> Cl <sup>b</sup>	8 57	8 25
			CH <sub>3</sub> I	16	66 6	134-136	C <sub>16</sub> H <sub>21</sub> N <sub>2</sub> O <sub>4</sub> I <sup>e</sup>	6 48	6 32

<sup>a</sup> Boiling points at 0.3-0.5 mm pressure, unless otherwise indicated. <sup>b</sup> Recrystallized from an absolute ethanol anhydrous ether mixture. <sup>c</sup> From absolute ethanol. <sup>d</sup> From absolute methanol anhydrous ether mixture. <sup>e</sup> No solvent was found satisfactory to recrystallize this compound. <sup>f</sup> A viscous oil which could not be distilled at 0.3-0.5 mm without decomposition. <sup>g</sup> Melting point. <sup>h</sup> Extremely hygroscopic. <sup>i</sup> Nitrogen analyses by a semimicro Kjeldahl method, unless otherwise indicated. <sup>j</sup> Analysis performed by Galbraith Laboratories, Knoxville, Tenn. <sup>k</sup> Melting points are uncorrected and were taken in capillary melting point tubes. <sup>l</sup> Melting point taken in a closed capillary tube. <sup>m</sup> B p at 1 mm pressure. <sup>n</sup> The free aminoester reported by Marszak, I, *et al*, *Compt rend*, 226, 1289(1948), b p 94° (0.5 mm). <sup>o</sup> *Ibid*, reported b p 163° (0.6 mm).

TABLE II.—1,4-BIS-N-SUBSTITUTED AMINO-2-BUTYNES



R	Yield, %	B.p. <sup>a</sup> °C	Salt	No	Yield, %	M.p. <sup>b</sup> °C	Formula	Analysis of N, %	
								Calcd	Found
Diethylamino <sup>c</sup>	75 0	73-79	Di-HCl	17	63 5	204-205	C <sub>12</sub> H <sub>26</sub> N <sub>2</sub> Cl <sub>2</sub> <sup>e</sup>	10 40	10 06
			Di-CH <sub>3</sub> I	18	91 3	213 5-214	C <sub>14</sub> H <sub>30</sub> N <sub>2</sub> I <sub>2</sub> <sup>f</sup>	5 83	5 51
Pyrrolidino <sup>d</sup>	92 5	98-102	Di-HCl	19	74 8	222	C <sub>12</sub> H <sub>22</sub> N <sub>2</sub> Cl <sub>2</sub> <sup>e</sup>	10 56	10 49 <sup>g</sup>
			Di-CH <sub>3</sub> I	20	80 5	246	C <sub>14</sub> H <sub>26</sub> N <sub>2</sub> I <sub>2</sub> <sup>h,i</sup>	5 88	5 85 <sup>j</sup>

<sup>a</sup> Boiling points at 0.3-0.5 mm pressure. <sup>b</sup> All melting points with decomposition. <sup>c</sup> Reported b p 110° (10 mm), Wille, F, *et al*, *Ann*, 591, 177(1955). <sup>d</sup> Reported b p 93-95° (0.1 mm), Biel, J, and DiPierro, F, *J Am Chem Soc*, 80, 4609(1958). <sup>e</sup> Recrystallized from anhydrous ether methanol mixture. <sup>f</sup> From absolute ethanol. <sup>g</sup> From absolute methanol. <sup>h</sup> Reported m p 239-240°, ref, see footnote d. <sup>i</sup> Analyzed by Galbraith Laboratories, Knoxville, Tenn.

## ANTIFUNGAL EVALUATION

The compounds were tested *in vitro* against *Trichophyton rubrum*, *Microsporum gypseum*, and *Microsporum audouinii*. The testing procedure was similar to that described by Goettseh (7), with the exceptions that aqueous solutions of the compounds and a seven-day incubation period were employed. At the end of the incubation period, the minimum zone of inhibition was measured. The zone of inhibition was the distance between the periphery of the paper disk and the colony growth. Compound 21, sodium undecylenate, was used as the control. The results of this antifungal study are given in Table III. Each value in this table represents an average of four zones of inhibition.

## DISCUSSION AND SUMMARY

The intermediates 4-chloro-2-butyne-1-ol and 1,4-dichloro-2-butyne were prepared by the procedure described by Bailey and Fujiwara (8). 4-Diethylamino-2-butyne-1-ol and the majority of the esters were prepared by the general procedures described by Biel, *et al* (6). The acetate and propionate esters were prepared by employing an excess of methyl acetate or methyl propionate without the use of the Dean-Stark water separator. The diamines were prepared by reacting an excess of the secondary amine with 1,4-dichloro-2-butyne.

The compounds deleted from Table III were inactive against all of the test organisms, which includes all of the 1,4-bis-N-substituted amino-2-butyne.



TABLE 111 —WIDTH OF ZONE OF INHIBITION IN MILLIMETERS (AVERAGE OF FOUR TESTS)

Compound Number	Name	Concn., %	<i>T. rubrum</i>	<i>M. gypseum</i>	<i>M. audouinii</i>
2	4-Diethylamino-2-butynyl hexanoate HCl	5.0	6.3	2.0	5.3
		2.5	2.8	0.0	3.0
		1.25	0.0	0.0	0.0
3	4-Diethylamino-2-butynyl octanoate CH <sub>3</sub> I	5.0	10.3	3.8	9.5
		2.5	3.5	1.3	8.0
		1.25	0.0	0.0	0.0
4	4-Diethylamino-2-butynyl crotonate HCl	5.0	2.0	0.0	1.0
		2.5	0.0	0.0	1.0
		1.25	0.0	0.0	0.0
5	4-Diethylamino-2-butynyl 10-undecenoate CH <sub>3</sub> I	5.0	15.5	13.7	"
		2.5	11.8	6.8	30.5
		1.25	11.8	3.3	13.8
6	4-Diethylamino-2-butynyl sorbate HCl	5.0	1.0	30.2	10.5
		2.5	0.0	15.5	9.5
		1.25	0.0	7.2	0.0
7	4-Diethylamino-2-butynyl sorbate CH <sub>3</sub> I	5.0	0.0	0.0	0.7
		2.5	0.0	0.0	0.0
		1.25	0.0	0.0	0.0
8	4-Diethylamino-2-butynyl benzoate HCl	5.0	13.5	9.5	7.3
		2.5	6.5	0.0	1.5
		1.25	0.0	0.0	0.0
10	4-Diethylamino-2-butynyl <i>o</i> -toluate HCl	5.0	"	31.5	30.5
		2.5	36.0	27.8	28.3
		1.25	14.0	17.3	21.5
11	4-Diethylamino-2-butynyl <i>o</i> -toluate CH <sub>3</sub> I	5.0	0.5	0.0	1.0
		2.5	0.0	0.0	0.0
		1.25	0.0	0.0	0.0
12	4-Diethylamino-2-butynyl <i>m</i> -toluate HCl	5.0	9.5	22.7	26.2
		2.5	0.0	11.7	17.5
		1.25	0.0	6.5	12.5
13	4-Diethylamino-2-butynyl <i>m</i> -Toluate CH <sub>3</sub> I	5.0	0.0	0.0	4.3
		2.5	0.0	0.0	0.5
		1.25	0.0	0.0	0.0
14	4-Diethylamino-2-butynyl <i>p</i> -toluate HCl	5.0	23.0	19.5	23.8
		2.5	17.5	7.5	15.0
		1.25	0.0	4.0	12.5
15	4-Diethylamino-2-butynyl <i>p</i> -nitrobenzoate HCl	5.0	16.5	8.8	22.0
		2.5	13.0	2.8	10.0
		1.25	7.5	0.0	0.8
21	Sodium undecylenate	5.0	25.0	18.0	37.5
		2.5	25.0	13.5	33.8
		1.25	20.3	6.0	30.0

• Complete inhibition

It was interesting to note that the aminoesters in which both the hydrochloride and methiodide salts were prepared, the hydrochlorides exhibited greater activity than their respective methiodide salts. This correlation was very evident in the benzoate, *p*-nitrobenzoate, and the *o*- and *m*-toluate esters, where their methiodide salts showed little or no activity, while the hydrochloride salts were all of reasonably good antifungal activity. This may be due to the fact that the hydrochloride salts possess a higher degree of water solubility than the methiodides, and thereby diffuse more rapidly across the agar plate surface which was covered with an aqueous spore suspension of the particular organism.

The only ester methiodide showing relatively good activity against all three organisms was the undecenoate ester methiodide. The carboxylic acid portion of the ester was probably an influential factor in this case.

In general, the ester hydrochlorides exhibited activity in the following order: aromatic > alkenyl > alkyl. In this classification, 4-diethylamino-2-butynyl *o*-toluate hydrochloride was superior to the others. It was also superior to sodium un-

decylenate against two of the fungi and showed excellent activity against the third. The excellent activity of this ester hydrochloride could be attributed to the steric effect produced by the *o* methyl group, thereby making the ester less vulnerable to hydrolysis and allowing a greater percentage of the compound to exert its activity in the form of the intact molecule.

Further antifungal studies and certain pharmacological testing will be performed on a number of these compounds at a later date.

## REFERENCES

- (1) Tanaka, K., Iwata, I., Okamura, Y., and Konotsune, T., *Antibiotics & Chemotherapy*, 9, 151(1959).
- (2) Imai, K., and Ikeda, N., *J. Pharm. Soc. Japan*, 76, 397, 400, 405(1956).
- (3) Grove, J. F., *Ann. Appl. Biol.*, 35, 37(1948).
- (4) Murhead, I., *ibid.*, 36, 250(1949).
- (5) Marszał, I., Marszał, Fleury, A., Ipszstein, R., Guermont, J., Jacob, J., and Montezin, G., *Mém. serv. clin. Hôp. Paris*, 36, 411(1951).
- (6) Biel, J. H., Sprengeler, F. P., and Friedman, H. I., *J. Am. Chem. Soc.*, 79, 6184(1957).
- (7) Goettlich, R., and Wiese, G., *This Journal*, 47, 319(1958).
- (8) Bailey, W. J., and Iijima, I., *J. Am. Chem. Soc.*, 77, 165(1955).

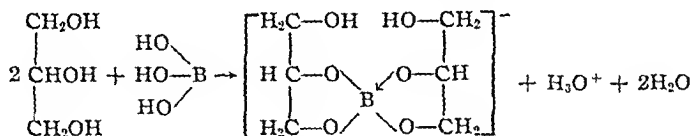
# A Solubility Study of the Boric Acid-Glycerin Complex I\*

## Solubility of Boric Acid in Glycerin Solution at 25°

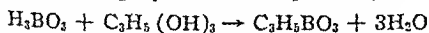
By JOHN J. SCIARRA and DONALD ELLIOTT

A solubility study of the boric acid-water-glycerin system was made at 25°. The effect of concentration of glycerin upon the solubility of boric acid was noted. Concentrations of glycerin ranging from 0 per cent to 100 per cent, by weight, were utilized in this study. A phase diagram of this three-component system was constructed at 25°. The results of this investigation were compared to the boric acid-water-sorbitol system and a difference was noted between the nature of the boric acid-sorbitol complex and the boric acid-glycerin complex.

GLYCERIN has been used for many years as a solvent for boric acid. This solvent allows the use of approximately 20 per cent of boric acid whereas a saturated solution of boric acid in water contains about 5 per cent of boric acid. Since 1905, the U. S. P. monograph (1) on boric acid has specified the use of glycerin in the chemical assay. The presence of glycerin allows boric acid to be titrated with sodium hydroxide as a monobasic acid (2). Previous investigators have established that a complex compound is formed between glycerin and boric acid although the exact nature of the complex has not been definitely established. This reaction may be represented as follows (3):



If a mixture of five moles each of boric acid and glycerin is heated to drive off all the water formed during the reaction, then a reaction represented by the following equation takes place (4):



This reaction takes place in the preparation of boroglycerin glycerite, N. F., which is used for its antibacterial properties. The former reaction is indicated when boric acid is dissolved in glycerin. Holm (5), Miner and Dalton (6), and Sciarra, Autian, and Foss (7) reported the solubility of boric acid in glycerin. Sorbitol is also known to

form a complex compound with boric acid and a solubility study of this system has been reported in a previous publication (8). It is interesting to note that the solubility of boric acid increased as the concentration of sorbitol increased to a maximum solubility of about 19 per cent boric acid in a 70 per cent, by weight, sorbitol solution at 25°.

A saturated solution of boric acid in 70 per cent sorbitol solution can be diluted with water without the precipitation of boric acid. This is not true in the case of a saturated solution of boric acid in glycerin. The addition of water will cause a precipitation of boric acid from such a solution. The formation of a precipitate is one of the problems associated with the use of boroglycerin glycerite when it is diluted with water. Boric acid is formed by the hydrolysis of glyceryl borate. This difference in behavior between solutions of boric acid in glycerin and in sorbitol prompted the following investigation. The solubility of boric acid in various concentrations of glycerin was determined at 25°. From the data, the effect of glycerin upon the solubility of boric acid was noted and the results plotted by means of a phase diagram.

## EXPERIMENTAL

Glycerin, U. S. P., containing over 99%, by weight, of  $\text{C}_3\text{H}_5(\text{OH})_3$  was used throughout this study. Solutions of glycerin were prepared by diluting glycerin with varying quantities of distilled water so that concentrations of glycerin were obtained from 0 to 100% glycerin, by weight, in increments of 5%. The specific gravity of each of the solutions, as well as the specific gravity of saturated solutions of boric acid in glycerin, was determined at 25° by the pycnometric method (9). This information enables one to express the solubility of boric acid in several different ways.

The solubility of boric acid in each of the above glycerin solutions was determined by adding a few drops of sodium hydroxide solution until the glycerin solution was slightly pink to phenolphthalein. Then an excess of finely divided, crystalline boric acid, U. S. P., was added to the solvent which had been

\* Received January 10, 1959, from St. John's University, College of Pharmacy, Jamaica 32, N. Y.  
Presented to the AAAS, Washington, D. C., meeting, December 1958.  
This investigation was supported by a Summer 1958 research grant from St. John's University.

placed into a solubility tube. The tube was then placed into a constant temperature water bath and stirred continuously for twenty-four hours (previous experiments showed that equilibrium had been attained during this period). After allowing the undissolved particles to settle to the bottom of the solubility tube, two 25-ml portions of the clear, supernatant liquid were removed, placed into tared beakers, and quickly weighed. The amount of boric acid in these solutions was determined by assaying the solutions according to the U S P assay for boric acid (10).

At the same time, a 25 ml Gay-Lussac specific gravity bottle was filled with the saturated solution of boric acid in the glycerin solvent and the specific gravity determined at 25°.

Temperature remained constant at 25° ± 0.1° by using a Sargent, full visibility, constant temperature water bath.

### RESULTS

The results of the above experiments are given in Tables I and II. Table I shows the specific gravity of various glycerin solutions and saturated solutions of boric acid in glycerin solvent at 25° while Table II gives the solubility of boric acid in these same glycerin solvents at 25°. Figures 1 and 2 show these results graphically.

In order to plot a phase diagram of the boric acid-water-glycerin solutions, the percentage composition of each component of this three-component system was calculated and is given in Table III. The phase diagram of this system is shown in Fig. 3.

The results given in Tables I, II, and III represent the average of three determinations for each solvent system.

### DISCUSSION

It is significant to note that the glycerin-boric acid complex behaves quite differently from the previously studied sorbitol-boric acid complex. As can be seen

TABLE I—SPECIFIC GRAVITY OF BORIC ACID SOLUTIONS IN GLYCERIN AT 25°

Concentration of Glycerin, % w/w	Glycerin Solution	Saturated Solution Boric Acid
0	1.000	1.019
5	1.012	1.031
10	1.024	1.043
15	1.036	1.054
20	1.049	1.065
25	1.062	1.078
30	1.074	1.090
35	1.087	1.102
40	1.100	1.114
45	1.113	1.128
50	1.128	1.142
55	1.140	1.154
60	1.153	1.170
65	1.168	1.181
70	1.181	1.197
75	1.195	1.209
80	1.208	1.222
85	1.219	1.235
90	1.232	1.248
95	1.245	1.262
100	1.260	1.278

TABLE II—SOLUBILITY OF BORIC ACID IN GLYCERIN SOLUTIONS AT 25°

Concentration of Glycerin, % w/w	ml Solvent/Gm Solute	% by Weight	% by Volume
0	17.58	5.53	5.64
5	17.63	5.33	5.50
10	17.66	5.24	5.46
15	17.67	5.18	5.46
20	17.54	5.21	5.55
25	17.45	5.11	5.51
30	17.19	5.14	5.60
35	16.70	5.22	5.75
40	16.34	5.27	5.87
45	15.49	5.48	6.18
50	14.93	5.61	6.40
55	14.07	5.87	6.77
60	13.06	6.22	7.28
65	11.78	6.77	7.99
70	10.45	7.48	8.96
75	9.00	8.51	10.29
80	7.34	9.89	12.08
85	6.75	10.82	13.36
90	5.19	13.51	16.86
95	4.20	16.04	20.24
100	3.29	19.40	24.79

TABLE III—COMPOSITION OF BORIC ACID-WATER-GLYCERIN SYSTEM AT 25°

Boric Acid % w/w	Glycerin % w/w	Water % w/w
5.53	0.00	94.47
5.33	4.73	89.94
5.24	9.48	85.28
5.18	14.22	80.60
5.26	18.95	75.79
5.11	23.72	71.17
5.14	28.46	66.40
5.22	33.17	61.61
5.27	37.89	56.84
5.48	42.53	51.99
5.61	47.20	47.19
5.87	51.77	42.36
6.22	56.27	37.51
6.77	60.60	32.63
7.48	64.76	27.76
8.51	68.62	22.87
9.89	72.09	18.02
10.82	75.80	13.38
13.51	77.84	9.65
16.04	79.76	4.20
19.40	80.60	0.00

from Fig. 1, the curves representing specific gravity of glycerin solvent (A) and specific gravity of a saturated solution of boric acid in the glycerin solvent (B) are separated throughout by essentially a constant. When sorbitol is used as a solvent for boric acid, this difference becomes less and less until finally both curves meet at approximately the specific gravity of a saturated solution of boric acid in 70% sorbitol solution. This seems to indicate that increasing the concentration of glycerin, up to a certain point, does not substantially increase the solubility of boric acid. This can be seen further by observing the essentially straight line from 0% to about 45-50% glycerin solutions in Fig. 2. The slight difference in results is probably due to experimental error. It is interesting to note that the presence of glycerin in these concentrations does not increase the solubility of boric acid. In fact the concentration of boric acid in this range of glycerin solutions is essen-

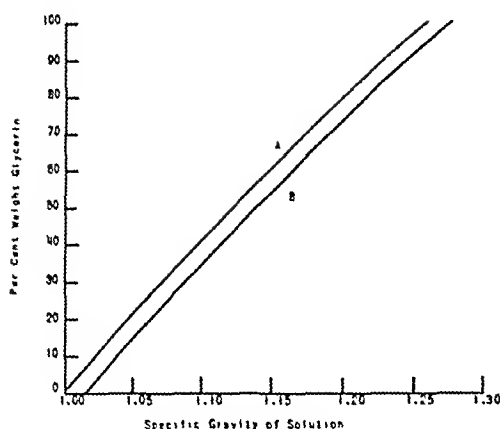


Fig. 1.—Specific gravity of boric acid-glycerin solutions at 25°. A, Glycerin solution; B, saturated solution of boric acid in glycerin.

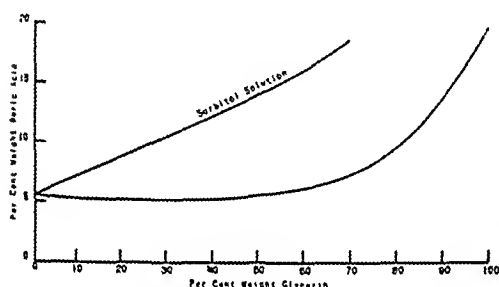


Fig. 2.—Solubility of boric acid in glycerin solutions at 25°.

tially the same concentration of boric acid as found in a saturated solution of boric acid in water. This observation leads one to believe that very little, if any, complex formation, between boric acid and glycerin takes place at this range in concentration of glycerin. Further investigations are indicated to definitely establish this observation. As can be seen from Fig. 2, as the concentration of glycerin is increased beyond 50%, the solubility of boric acid in the solvent increases.

It has previously been established that this increase in solubility is due to the formation of a boric acid-glycerin complex. This complex has a greater solubility than boric acid in the respective solvent system. However, the addition of water to these solutions will cause a precipitation of boric acid, probably due to a change of solvent, resulting in a decrease in glycerin concentration. As has been noted previously, this precipitation does not take place with saturated solutions of boric acid in sorbitol solutions. This indicates the increased stability of the boric acid-sorbitol complex as compared to boric acid-glycerin complexes. Preliminary experiments indicate that propylene glycol reacts similarly to glycerin, which is not surprising when one considers

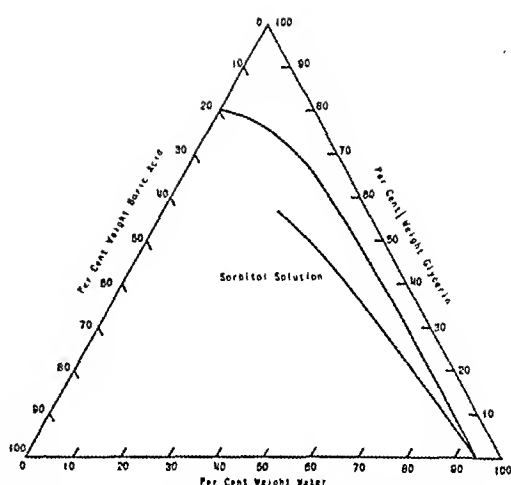


Fig. 3.—Phase diagram of boric acid-water-glycerin system at 25°.

the similarity of these two solvents. The results of this investigation suggest the possible replacement of the boroglycerin glycerite with a similar preparation utilizing sorbitol in place of glycerin, especially when a stock solution of boric acid is indicated. Bacteriological studies may indicate the possible use of this preparation for its antibacterial properties.

The phase diagram of the boric acid-water-glycerin system, Fig. 3, indicates the area of complete miscibility of these three components. This is represented by the area above the curve. As can be seen, the area above the sorbitol curve is greater, indicating a greater area of miscibility for the sorbitol system as compared to the glycerin system. Figure 3 can be used to determine the minimum amount of glycerin necessary to dissolve a given amount of boric acid. This information is essential when one considers problems of formulation.

## REFERENCES

- (1) "United States Pharmacopeia," 8th rev., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 8.
- (2) Cook, F. E., and Martin, E. W., "Remington's Practice of Pharmacy," 11th ed., Mack Publishing Co., Easton, Pa., 1956, p. 1235.
- (3) Soine, T. O., and Wilson, C. O., "Roger's Inorganic Pharmaceutical Chemistry," 6th ed., Lea and Febiger, Philadelphia, Pa., 1957, p. 122.
- (4) Cook, F. E., and Martin, E. W., "Remington's Practice of Pharmacy," 11th ed., Mack Publishing Co., Easton, Pa., 1956, p. 518.
- (5) Holm, Pharm. Weekblad, 58, 860(1921); through Chem. Abstr., 15, 2962(1921).
- (6) Miner, C. S., and Dalton, N. N., "Glycerol," Reinhold Publishing Co., New York, N. Y., 1953.
- (7) Sciarrra, J. J., Autian, J., and Foss, N. E., J. Am. Pharm. Assoc. Pract. Pharm. Ed., 18, 366(1957).
- (8) Sciarrra, J. J., Autian, J., and Foss, N. E., THIS JOURNAL, 47, 144(1958).
- (9) Daniels, F., Mathews, J. H., and Williams, J. W., et al., "Experimental Physical Chemistry," 5th ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1956, p. 375.
- (10) "U. S. Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 103.

# Comprehensive Studies on Utah-Grown Medicinal Rhubarb<sup>+</sup>

By R. L. WORKMAN, Jr., and L. D. HINER

The rhizomes and roots of medicinal rhubarb grown in Utah were subjected to the N F X tests for rhubarb. In addition to the official tests, tests for rhaponticin, laxative activity, and organoleptic acceptance were performed. The results of the combined tests indicated that the Utah-grown medicinal rhubarb possessed satisfactory laxative properties, and acceptable taste and odor qualities.

**R**HUBARB has been known and used as a medicine for over forty five hundred years. The first written reference to rhubarb was made in the Chinese herbal "Pen King" which has been attributed to the Emperor Chen nung (1) and was written about 2700 B C. Dioscorides referred to *rha* or *rheon* during the first century A D. *Rha* was the Latin word for the Volga River, and it is possible that the *rha* of that period was rhapontic rhubarb, *Rheum rhaponticum*, from the Black Sea area, although Chinese rhubarb may have reached Europe as early as the first century A D (2). The Arabs knew and used rhubarb during the Middle Ages. They were aware that the drug which came from the East via Persia was of Chinese origin and preferred it to the "pontic" variety (3). This preference for Chinese rhubarb has remained until the present time. Indeed, the National Formulary IX imposed a geographic limitation for official rhubarb to China and Tibet (4) and the National Formulary X further restricted this source to China (5).

Because of the source limitation imposed in the N F, the supply of rhubarb to the United States has always been subject to the political condition of the Far East. This uncertainty of supply has led to investigations of other sources for this medicinal agent. Indian rhubarb (*Rheum emodi* and *Rheum acbbarum*) was offered for importation, and after careful study, was admitted to the National Formulary in 1950 as a substitute for Chinese rhubarb. Attempts to grow medicinal rhubarb in the United States have met with very little success. DeRose states that medicinal rhubarb will grow in Massachusetts, Minnesota, and Illinois,

but for some reason the medicinal rhubarbs grown in these areas reportedly have an emetic rather than a laxative action (3). Because of the difference in climate between Utah and other areas of the United States in which this drug has been grown, it was decided to investigate the possibility that medicinal rhubarb could grow in Utah that would have the desired laxative action.

The rhubarb used in this investigation was obtained from three sources. Hybrid *Rheum officinale* grown in Utah from seed, Utah grown *Rheum rhaponticum*, and rhubarb, N F obtained from the S B Pemick Company. The Utah medicinal rhubarb was grown from seed obtained from Dr. Heber W. Youngken of the Massachusetts College of Pharmacy. The seed was planted in the greenhouse in the fall of 1948. The plants obtained from this seed were moved into the field during the spring of 1949. Between 1949 and 1956 these plants were virtually unattended, but still thrived. During the last three growing seasons (1956, 1957, and 1958) the plants were brought under cultivation and finally, the roots and rhizomes were harvested October 21, 1958. The Utah grown rhaponticum was harvested at approximately the same time. After harvesting, the roots and rhizomes of both species were washed with water and then carefully peeled. The peeled roots and rhizomes were then placed in a warm room to dry. The official N F rhubarb samples were of Chinese origin, and were in granulated and whole commercial forms.

## PROCEDURES

**Microscopic.**—Samples of dried roots of Utah grown medicinal rhubarb, Utah grown *Rheum rhaponticum*, and Chinese medicinal rhubarb were placed in 50% ethyl alcohol and left overnight. Normal dehydrating procedures were then used to prepare the samples for embedding in paraffin. Sections made using a rotary microtome were made into permanent mounts and examined with the aid of a research microscope. Samples of the dried roots and rhizomes of all three rhubarbs were ground in a Wiley mill to a number forty powder. They were then examined microscopically using chloral, phloroglucin hydrochloride, glycerine, and iodopotas iodide solution as reagents.

**Pharmaceutical Preparations.**—The preparations used in the organoleptic comparisons and the pharmacological tests were made according to the directions in the official literature. In the case of el

\*Received August 21, 1959 from the College of Pharmacy, University of Utah, Salt Lake City.

Abstracted from a thesis submitted by R. L. Workman, Jr. in partial fulfillment of the degree of Doctor of Philosophy to the University of Utah, Salt Lake City.

Presented at the meeting of the American Pharmaceutical Association, Section A, Philadelphia, August 1959.

Supported in part by a research grant from the University of Utah Research Fund.

of the preparations, which included the fluidextract, aromatic tincture, and aromatic syrup, one set of samples was made using official rhubarb and one set using the Utah-grown hybrid.

**Tests.**—The tests conducted during this investigation consisted of the official tests as listed in N. F. X, some nonofficial but established tests for rhaponticin, and organoleptic comparisons. Official commercial rhubarb was used as the control for the official tests and organoleptic comparisons, while *Rheum rhaponticum* was used as the control for the nonofficial tests for rhaponticin.

**Laxative Activity.**—Albino mice were used as the test animals in the laxative activity test procedure which was the same as that used for senna and cascara (6, 7, 8). Animals weighing between 20 and 30 Gm. were placed in individual wire cages which were placed upon newsprint. The animals were allowed access to a mixture of powdered Rockland mouse diet and water, mixed in the ratio of 1 Gm. of the laboratory diet to 2 cc. of water, during the entire screening and experimental period. At the end of two hours the newsprint was examined for signs of diarrhea, as shown by sufficient moisture in the stool to stain the underside of the paper. Animals showing signs of diarrhea were discarded and not used in this investigation. The remaining animals were then divided into four groups of 15 animals each.

Two rhubarb fluidextracts were used as experimental drugs: rhubarb fluidextract N. F. and rhubarb fluidextract made with medicinal rhubarb grown in Utah. These fluidextracts were mixed with water in appropriate amounts so that each animal received the desired amount of fluidextract in a volume of 10 cc./Kg. of body weight. This formula resulted in a dosage sufficiently large to permit accurate and ready measurement. All doses were given orally using a hypodermic syringe and a hypodermic needle from which the point had been removed and solder placed on the end to prevent injury to the animal during the administration of the medication.

Six hours after the administration of the drug, the newsprint beneath the cages was checked for signs of diarrhea. If there were signs of diarrhea the dose of the drug for that animal was considered positive for that particular animal. If there were no signs of diarrhea, the dose of the drug for that particular animal was considered negative. The laxative activities of the two fluidextracts were compared statistically by the method Miller (9) employed to compare the laxative activity of various samples of senna and the cardiac activity of various samples of digitalis.

## EXPERIMENTAL

The medicinal rhubarb plants grown in Utah for this investigation at first were thought to be *Rheum officinale* because of information supplied with the seed. Later, however, they would not check out as this species when compared to available keys. Specimens of the leaves and stems with the inflorescence were sent to Dr. Heber W. Youngken. With his cooperation it was determined that the plants used in this investigation were a hybrid of *Rheum officinale* and *Rheum tartaricum* (10).

The roots and rhizomes were examined after they had become completely dry. The dried, unground rhizomes and roots were cylindrical in form, from

6–10 cm. in length and from 1.5–5 cm. in diameter. The outer surface was yellowish brown with patches of brown cork and partially covered with yellow powder. The roots and rhizomes were hard and compact. The fracture was uneven and granular. The newly-fractured surface was pinkish brown in color and, upon standing, a yellow powder formed on this surface. The odor was aromatic and characteristic. The taste was bitter and astringent.

Cross sections of the roots of Utah medicinal rhubarb were compared with cross sections of Chinese rhubarb and rhapontic rhubarb. All three exhibited regular dicotyl structure. The cross sections were devoid of cork cells and cortex because all of the roots were peeled previous to the preparation of the slides. The phloem and cambium were not very pronounced. The xylem was radiate, while the medullary rays were wavy and mostly two cells in width. The tracheae were scattered and in small groups. The xylem consisted of wood parenchyma and contained starch, calcium oxalate rosette aggregate crystals, and some tannin.

The powder of the Utah medicinal rhubarb was of a moderate yellowish-brown color. Microscopic examination of the powder revealed calcium oxalate rosette aggregates from about 40–120  $\mu$  in diameter. Starch grains 5–20  $\mu$  in diameter, grouped in two's and four's, bunched and single with central cleft hilums were present. There were fragments of vascular rays containing a yellow substance which was soluble in water, insoluble in alcohol, and soluble in ammonia T. S., which turned the substance pink. The vessels were nonlignified, mostly reticulate, with a few elements being spiral.

One per cent potassium hydroxide solution turned both the Utah medicinal rhubarb and the Chinese rhubarb powders a very dark red, which indicated the presence of anthraquinone compounds. The N. F. test for emodin and chrysophanic acid was positive for both rhubarb powders. The Utah medicinal rhubarb contained 35% diluted alcohol-soluble extractive while the official sample contained 39%. Both of these figures are well above the N. F. minimum of 30% diluted alcohol-soluble extractive.

Utah medicinal rhubarb and Chinese rhubarb were tested for rhaponticin while rhapontic rhubarb was used as a control. One-half gram of each powdered rhubarb was shaken with 5 cc. of 2% ammonia solution and allowed to stand in a warm room for fifteen minutes. They were then filtered, and each filtrate was placed on a watch glass. Almost immediately the filtrate from the rhapontic rhubarb had crystals formed in it. At the end of four hours neither the filtrate from the Chinese rhubarb nor the filtrate from the Utah medicinal rhubarb had any signs of crystal formation.

Powdered samples of the three rhubarbs were placed under ultraviolet light to see if rhaponticin could be detected by this means. The rhapontic rhubarb appeared light in color and had a weak purple fluorescence. Both the Utah medicinal rhubarb and the Chinese rhubarb were dark in color, and what little fluorescence was present appeared very dark brown.

Organoleptic tests were performed by a forty-five-member panel consisting of faculty, graduate students, and seniors. The fluidextracts, aromatic tinctures, and aromatic syrups were compared for

color, odor, taste, and preference. Sixty-seven per cent of the panel preferred the fluidextract made with the Utah medicinal rhubarb and one panel member reported that the fluidextract made with the Chinese rhubarb had nauseating qualities. The aromatic tincture made with the Utah medicinal rhubarb was preferred by 67% of the panel and one member of the panel reported nauseating qualities in this aromatic tincture, while another panel member reported these qualities in the aromatic tincture made with the Chinese rhubarb. Sixty-seven per cent of the panel preferred the aromatic syrup made with the Chinese rhubarb. One panel member reported nauseating qualities present in this aromatic syrup.

Two comparison tests were made to determine the laxative activity of the two fluidextracts. The first test compared the laxative activity for both fluidextracts in doses of 0.5 cc./Kg. of body weight and 1 cc./Kg. of body weight. The second comparison was run independent of the first, and the doses employed were 0.25 cc./Kg. of body weight and 0.5 cc./Kg. of body weight. The results obtained in the two experiments were statistically compared by the method outlined by Miller (9). The laxative activity of the rhubarb fluidextract N. F. was arbitrarily assigned a value of one and the fluidextract made from the Utah medicinal rhubarb was compared with it. In the first test the Utah fluidextract had a value of 0.72 with 95% fiducial limits of 0.39 and 1.03, while in the second test this fluidextract had a value of 0.88 with 95% fiducial limits of 0.48 and 1.28. The results were also combined, and a final statistical analysis was made of the combined data obtained with the two fluidextracts. The potency of the N. F. fluidextract was again assigned the value of one and in this analysis the Utah fluidextract had a weighted mean potency of 0.80 with 95% fiducial limits of 0.55 and 1.05. In all three instances the upper 95% fiducial limit overlapped the value of one assigned to the N. F. fluidextract and one must

conclude that the two fluidextracts have the same potency.

## SUMMARY

1. A hybrid of *Rheum officinale* and *Rheum tartaricum* has been grown in Utah. It grew even when left virtually unattended for a period of six years.

2. Macroscopic and microscopic examination reveals that this hybrid is very similar to commercial samples of Chinese rhubarb and fits the description of rhubarb given in the N. F. X.

3. This hybrid compares favorably with commercial samples of Chinese rhubarb when subjected to the N. F. X tests for rhubarb.

4. Pharmaceutical preparations made with this hybrid are acceptable from an organoleptic standpoint and are relatively free of objectionable qualities.

5. The laxative potency of at least one of its preparations appears to be the same as that of the corresponding official preparation.

## REFERENCES

- (1) Wallis, T. E., "Textbook of Pharmacognosy," J. and A. Churchill Ltd., London, England, 1955, p. 356.
- (2) Claus, E. P., "Gathercoal and Wirth Pharmacognosy," 3rd ed., Lea & Febiger, Philadelphia, Pa., 1956, p. 135.
- (3) DeRose, A. F., and Wirth, E. H., *Pharm. Arch.*, 15, 65(1944).
- (4) "The National Formulary," 9th ed., The American Pharmaceutical Association, Washington, D. C., 1950, p. 430.
- (5) "The National Formulary," 10th ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 483.
- (6) Grote, I. W., and Woods, M., *THIS JOURNAL*, 33, 266(1944).
- (7) Miller, L. C., and Alexander, E. B., *ibid.*, 38, 417(1949).
- (8) Lish, P. M., and Dungan, K. W., *ibid.*, 47, 371(1958).
- (9) Miller, L. C., *ibid.*, 33, 245(1944).
- (10) Youngken, H. W., Personal communication, August 3, 1958.

## Book Notices

*Recent Progress in Oxytocin Research.* By B. BERDE. Charles C Thomas, 301 East Lawrence Ave., Springfield, Ill., 1959. ix + 110 pp. 14 x 21.5 cm. Price \$4.75.

The extent of accepted knowledge of oxytocin and its definite physiological functions is indicated by the fact that this good review occupies 82 pages. The alphabetically arranged author references (starting with Abel, J. J.) cover 22 pages. Particularly interesting is the second chapter which discusses the relationship between the chemical structure of oxytocin and its biological activity.

*Lehrbuch der organischen Chemie.* By PAUL KARRER. Georg Thieme Verlag, Herdweg 36, Stuttgart, N. Germany, 1959. xx + 1057 pp. 17.5 x 26 cm. Price DM 60.

This is the thirteenth edition of a classical textbook (in German) an organic chemistry. The book has retained its established style and arrangement, but its text has been considerably revised since the publication of the twelfth edition, which was reviewed in *THIS JOURNAL*, 43, 384(1954). Earlier editions have appeared in English translations and it is hoped that this edition also will be translated.

JOURNAL OF THE  
AMERICAN PHARMACEUTICAL  
ASSOCIATION

VOLUME 49

MARCH 1960

NUMBER 3

## Enteric Coatings III\*

An Improved Enteric Coating and Its *In Vitro* Evaluation

By JOHN G. WAGNER and STUART LONG

A new, improved enteric coating, prepared from styrene-maleic acid copolymer, dibutyl phthalate, and talc (I) was compared with previously reported coatings containing starch acetate phthalate, dibutyl phthalate, and talc (II), and cellulose acetate phthalate, propylene glycol, talc, and sorbitan monooleate (III). Increasing the amount of talc applied per tablet increases the disintegration time of the coated tablets in artificial intestinal fluid. This effect is least for I, intermediate for II, and greatest for III. A minimum amount of talc is essential to obtain satisfactory acid resistance with I but an increase in the amount of talc does not confer increased acid resistance. The ratio of gastric resistance time : disintegration time in artificial intestinal fluid is greater for I than for II. The ratio of disintegration time in artificial intestinal fluid : weight of enteric coating is much less for I than for either II or III. These observations clearly indicate advantages of I over II and III as enteric coatings. The types of plots presented are useful for comparing one type of coating with another and predicting the properties of the different coatings.

VARIOUS DEFINITIONS of an efficient or suitable enteric coating have appeared in the literature (1, 2). It is proposed that the ideal enteric coating is one which would: (a) not disintegrate in the stomach in the duration of time that the enteric coated dosage form may be expected to remain in the stomach; (b) release the contained drug immediately upon reaching the upper small intestine; (c) have a disintegration time *in vitro* and *in vivo* in intestinal fluids which is essentially independent of the thickness or weight of the coating; (d) show no change in properties under normal or accelerated storage conditions; (e) be nontoxic; (f) be easy to apply; and (g) be economical. Such ideals are probably impossible to attain in practice but the closer a given coating approaches them, the better the coating.

From a practical standpoint it is more important to ensure that an enteric coated dosage form of many drugs will liberate the contained drug at the major site of absorption of these drugs, namely the duodenum and upper small intestine, than it is

to ensure that the coating will remain intact in the stomach for many hours.

In this communication, the third in the series (3, 4), the *in vitro* evaluation of a new improved enteric coating is reported. This coating was recently disclosed (5). It was discovered that styrene-maleic acid copolymer forms excellent enteric films in combination with a micaceous mineral solid. Usually a plasticizer is also incorporated into the enteric film. Coatings prepared from styrene maleic acid (SY-MA) copolymer, di-*n*-butyl phthalate, and talc have excellent enteric properties in the dog<sup>1</sup> and human beings. Results obtained in human subjects will be reported in a subsequent communication. One of the more interesting aspects of this coating is its relatively high resistance *in vitro* up to pH 2.6 and its high susceptibility to breakdown at and above pH 3.4; this unique property will be discussed subsequently, also.

Very little investigation of the effect of dusting powders on the properties of enteric coatings has

\* Received August 13, 1959, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

<sup>1</sup> Lots I and III of the previous publication (3) were the same as lots B1-1 and BIII-2, respectively, in this publication.



been reported in the literature. It seems to have always been assumed, until recently, that the powder was inert and contributed little to the disintegration behavior of the coating. Hawkins and Thompson (2) investigated the effect of a large number of dusting powders, each at one concentration level, with three different enteric coating solutions. Since only one concentration level was used in each case, it was impossible to determine what real effect each dusting powder had on the properties of each of the coating compositions. A British patent (6) disclosed that dispersion of a finely divided mineral solid in an enteric film-forming substance, such as cellulose acetate phthalate, rendered the coatings substantially free from cracking and crazing upon aging. We have found that the concentration level of talc in three modern enteric coatings is an important factor in determining the disintegration characteristics of the resultant coatings.

Reports concerning enteric coatings in the past have always stressed the differences in results achieved with the various enteric substances. In this communication it is stressed that one should not compare enteric substances unless one takes into consideration the amounts of the enteric substance and the dusting powder employed. Also certain empirical relationships which are useful in predicting the properties of enteric coatings and in comparing one type of coating with another are presented.

## EXPERIMENTAL

**Materials.**—The styrene-maleic acid copolymers used were obtained by complete hydrolysis of styrene-maleic anhydride copolymers. The latter were prepared by conventional polymerization techniques (7, 8, 9). The type 1 copolymer had a considerably greater molecular weight than the type 2 copolymer. Further details may be found in reference (5). The starch acetate phthalate was S.A.P. III described in the previous publication (4) of this series. The cellulose acetate phthalate was described previously (4). Other materials were: di-*n*-butyl phthalate #1403 (Eastman Organic Chemicals), methyl ethyl ketone (Shell Development Corp.), and U. S. P. supreme talc (Sierra Talc and Clay Co.).

**Enteric Coating.**—The properties of the compressed and subcoated tablets prior to enteric coating are shown in Table I. The subcoated tablets were coated with different enteric coating solutions (see Table II) by conventional pan coating techniques. In most cases talc was used as a dusting powder.

In study A, two lots of subcoated tablets were enteric coated with solution AI and two lots of the same subcoated tablets were enteric coated with solution AII. Talc was used as a dusting powder on one lot coated with solution AI and on one lot coated AII. No dusting powder was applied to the

second lots coated with solutions AI and AII. The tablets of all four lots were coated with an average of 30.9 mg. of SY-MA copolymer per tablet. For the lots coated with talc and solutions AI and AII, the average weight of the enteric coating per tablet was 125 and 127 mg., respectively.

In study B, lots of subcoated tablets were coated with coating solutions BI and BII shown in Table II and with solution BIII, containing cellulose acetate phthalate. Solution BIII was prepared according to example 1 of reference (6). In study B the weight of enteric polymer applied per tablet was held essentially constant but the amount of dusting powder applied per tablet varied from lot to lot. Lots BI-1, BI-2, BII-1, and BII-2 were all coated with an average of 25 mg. of styrene-maleic acid copolymer per tablet. The weight of talc applied per tablet, however, varied from 91 to 147 mg. Lots BIII-1, -2, and -3 were coated with approximately the same amount of cellulose acetate phthalate per tablet, namely  $13.5 \pm 1.5$  mg. The weight of talc applied per tablet was varied from 49 to 88.7 mg.

In study C, lots of 10,000 subcoated tablets were coated with coating solutions CI, CII, and CIII (see Table II). After a definite amount of enteric coating had been applied some of the tablets of each batch were withdrawn and dried. In study C both the weight of enteric polymer and the weight of talc applied was increased from lot to lot for a given enteric polymer but the ratio of talc:enteric polymer remained essentially constant. For lot CI-1 through CI-6 this ratio averaged 3.09, with a range of 2.91 to 3.21. For lots CII-1 through CII-6 this ratio averaged 2.85, with a range of 2.5 to 3.05. For lots CIII-1 through CIII-5 this ratio averaged 2.71 with a range of 2.55 to 2.79.

**Enteric Coated Tablets.**—The resistances of the four lots of enteric coated tablets, which were coated in study A, to simulated gastric fluid U. S. P. and their disintegration times in artificial intestinal fluid, pH 6.9, are shown in Table III.

Tables IV and V give the properties of the enteric coatings produced in studies B and C, respectively. The thicknesses of the enteric coatings on the side and edge of the tablets of lots CI-1 through CI-6 are plotted in Fig. 7. These thicknesses were determined by individually measuring 100 enteric coated and 100 subcoated tablets of each lot with a micrometer reading to 0.001 inch.

*In vitro* disintegration tests were carried out using the apparatus, fluids, and end points described in a previous publication (3). In study C, some of the lots were tested in artificial intestinal fluid, pH 6.9 by two methods: (a) by placing the tablets directly into fluid, and (b) by putting the tablets in simulated gastric fluid U. S. P. for two hours then transferring them to the other fluid. As before (3) the average disintegration time of each enteric coating,  $T_e$ , in the pH 6.9 fluid was assumed to be the difference in the average disintegration time of the whole enteric coated tablet and the average disintegration time of the corresponding subcoated tablet. The average time to the coating attacked end point in simulated gastric fluid U. S. P.,  $T_g$ , was also determined as described previously (3). The standard errors,  $S_{T_e}$ ,  $S_{T_g}$ , and  $S_{T_d}$  of the averages  $T_e$ ,  $T_g$ , and  $T_d$ , respectively, are also given in Tables IV and V.

TABLE I.—PROPERTIES OF THE COMPRESSED AND SUBCOATED TABLETS PRIOR TO ENTERIC COATING

Lot of Enteric Coated Tablets	Compressed Tablet		Subcoated Tablet <sup>a</sup>				Av. Disintegration Time <sup>b</sup> ± S.E. <sup>c</sup> Min
	Av. Wt., Gm	Punch Size and Shape in	Av. Thickness, in Edge	in Side	Av. Wt., Gm	Av. Vol. cu mm	
CI-1 to -6; CII-1 to -6; CIII-1 to -5	0.418	3/8 oval	0.414	0.211	0.576	.	7.4 ± 0.6
BI-1 and -2; BII-1 and -2	0.426	3/8 oval	0.407	0.207	0.542	301	4.8 ± 0.2
BIII-1	0.266	3/8 oval	0.405	0.199	0.376	.	2.0
BIII-2 and -3	0.437	1/8 oval	0.403	0.214	0.543	305	8.8 ± 0.3

<sup>a</sup> The subcoatings were of the carboxymethyl cellulose type described in U. S. pat. 2,693,437 (1954)<sup>b</sup> Average disintegration time of the subcoated tablets in artificial intestinal fluid, pH 6.9, using the U. S. P. apparatus. The end point was taken when 99 to 100% of the entire tablet was through the screen of the disintegration apparatus<sup>c</sup> Standard error of the average

TABLE II — COMPOSITION OF COATING SOLUTIONS

Ingredient	Solution <sup>a</sup>						
	AI	AII	BI	BII	CI	CII	CIII
tyrene-maleic acid copolymer (type 1)			15.625	15.625		20	..
tyrene-maleic acid copolymer (type 2)	5	15			20		
tarch acetate phthalate							12.5
n-butyl phthalate	0.6	1.8	1.875	1.875	2.4	2.4	1.5
alcohol 3A, q. s.	✓	✓	✓		✓	✓	
qual parts of alcohol 3A and acetone, q. s.				✓			
fethyl ethyl ketone, q. s.							✓

<sup>a</sup> Concentration expressed as % w/v. Check marks indicate solvent used for particular solution

TABLE III — DISINTEGRATION BEHAVIOR OF TABLETS PRODUCED IN STUDY A

	Disintegration Tests, Time in Minutes		
	$T_i \pm S_{T_i}$	$T_c$	$T_d \pm S_{T_d}$
compressed tablet	0.5 ± 0		0.4 ± 0 <sup>a</sup>
subcoated tablet	4.5 ± 0.18	4.0	2.5 ± 0.19 <sup>a</sup>
enteric coated tablet (solution AI, no talc)	9.25 ± 0.62	4.75	12.8 ± 1.94 <sup>a</sup>
enteric coated tablet (solution AI + talc)	17.4 ± 1.00	12.9	{ 238 ± 40 <sup>a</sup> (180 ± 5.7) <sup>b</sup> } { >233 <sup>a</sup> (>202) <sup>b</sup> }
enteric coated tablet (solution AII, no talc)	8.75 ± 0.44	4.25	11.6 ± 0.92 <sup>a</sup>
enteric coated tablet (solution AII + talc)	17.7 ± 0.36	13.2	{ 251 ± 16 <sup>a</sup> (204 ± 10) <sup>b</sup> } { >300 <sup>a</sup> (255 ± 24) <sup>b</sup> }

<sup>a</sup> End point taken when 99 to 100% of tablet was through the screen of U. S. P. disintegration apparatus<sup>b</sup> Coating slightly attacked end point<sup>c</sup> Two separate tests. One test was terminated at six hours and since one or more tablets had not reached the end point the average could not be computed

**Evaluation of Enteric Coatings.**—Adequate evaluation of an enteric coating necessitates both *in vitro* and *in vivo* tests. Interpretation of the effect of different variables on the *in vitro* disintegration behavior of an enteric coating and comparison of different enteric coatings may conveniently be made by use of such plots as shown in Figs. 1 through 6 or a wide variety of enteric coatings the points on such plots can be fitted by the equations of the straight line,  $Y = ax + b$  or  $Y = ax$  or by a simple polynomial of the type  $Y = ax + bx^2$ . The slopes of the lines and their position characterize a given type of coating. Direct comparison of two different coating systems may also be made by comparison of the slopes of the lines obtained for these different systems on the same type of plot.

Values of disintegration time are slightly lower when the tablets are placed directly into artificial intestinal fluid than when they are tested in the same fluid following two hours in simulated gastric fluid. Practically, the difference is insignificant and in most cases a significant difference cannot be shown between two such individual values at a high level of confidence. However, when plotting the data the difference is important. When values of

disintegration time in artificial intestinal fluid following gastric fluid are plotted against such abscissas as average weight of enteric coating, average weight of enteric polymer, etc., the lines obtained cannot be extrapolated to pass through the origin. Usually such lines have a negative intercept (e.g., see Fig. 1). However, when the average disintegration times obtained by direct immersion in artificial intestinal fluid are plotted against similar abscissas, lines are obtained which do pass through the origin when extrapolated (see Figs. 3 through 6).

## DISCUSSION

Three separate but interrelated studies are reported in this communication.

Study A compared coatings prepared from SY-MA copolymer and dibutyl phthalate, with and without talc, at one level of SY-MA copolymer and one level of talc. The enteric polymer was applied to the tablets as solutions of two different concentrations.

Study B compared coatings prepared from SY-MA copolymer and cellulose acetate phthalate. For each enteric polymer lots of tablets were coated with constant amounts of the enteric substance but

TABLE IV.—PROPERTIES OF ENTERIC COATINGS PRODUCED IN STUDY B

Lot	Average Weight Applied/Tablet, mg		Average Volume, cu mm	Average Amount of Coating $\pm$ Standard Error			Disintegration Tests			Simulated Gastric Fluid, pH 1.2
	Enteric Polymer	Talc		Weight, <sup>a</sup> mg	Thickness, <sup>a</sup> Edge	Inches $\times 10^3$ Side	Intestinal, pH 6.9 After Gastric	T <sub>c</sub> $\pm$ S <sub>T<sub>c</sub></sub>	T <sub>c</sub>	
BI-1	25	91	40.0 $\pm$ 1.2	81 $\pm$ 3.5	8.8 $\pm$ 0.8	7.6 $\pm$ 0.5	15.1 $\pm$ 0.3	10.3	285	$\pm$ 26
BI-1-1	25	130	58.4 $\pm$ 3.1	138 $\pm$ 3.5	10.1 $\pm$ 0.5	10.9 $\pm$ 0.3	41.0 $\pm$ 0.4	36.2	232.5	$\pm$ 6.6
BI-1-2	25	129	65.0 $\pm$ 2.2	138 $\pm$ 3.1	10.1 $\pm$ 0.5	11.2 $\pm$ 0.4	41.3 $\pm$ 0.7	36.5	235	$\pm$ 6.1
BI-2	25	147	67.5 $\pm$ 1.9	150 $\pm$ 3.5	10.8 $\pm$ 0.4	11.5 $\pm$ 0.3	46.8 $\pm$ 0.3	42.0	225	$\pm$ 10.0
BI-1-1-1	13.4	49		45 $\pm$ 2.1	3.7 $\pm$ 0.2	3.3 $\pm$ 0.5	14.8 $\pm$ 0.5	12.8		...
BI-1-1-2	12	57.4	34.2 $\pm$ 1.5	73 $\pm$ 3.0	4.6 $\pm$ 0.3	6.3 $\pm$ 0.4	43.8 $\pm$ 0.6	35.0		> 120
BI-1-1-3	15	88.7	40.0	91 $\pm$ 3.1	6.5 $\pm$ 0.5	7.5 $\pm$ 1.0	60.0	51.2		..

<sup>a</sup> Based on 20 individually measured enteric coated and subcoated tablets

TABLE V.—PROPERTIES OF ENTERIC COATINGS PRODUCED IN STUDY C

Lot	Av. Wt. Applied Per Tablet, mg		Av. Wt. of Coating <sup>a</sup> mg	Disintegration Tests, Time in Minutes				Sim. Gastric Fluid, pH 1.2
	Enteric Polymer	Talc		Intestinal, pH 6.9 After Gastric	T <sub>c</sub> $\pm$ S <sub>T<sub>c</sub></sub>	Intestinal, pH 6.9 Directly	T <sub>c</sub> $\pm$ S <sub>T<sub>c</sub></sub>	
CI-1	10	32.1	33			11.8 $\pm$ 0.4	4.4 $\pm$ 0.4	68 $\pm$ 2.3
CI-2	15	46.4	47			13.6 $\pm$ 0.2	6.2 $\pm$ 0.3	110 $\pm$ 2.3
CI-3	19.7	59.8	60	14.8 $\pm$ 0.4	7.4 $\pm$ 0.4	14.2 $\pm$ 0.3	6.8 $\pm$ 0.3	125 $\pm$ 1.6
CI-4	24.7	77.8	75	17.3 $\pm$ 0.5	9.9 $\pm$ 0.5	16.2 $\pm$ 0.2	8.8 $\pm$ 0.3	158 $\pm$ 4.2
CI-5	29.7	86.4	84	19.1 $\pm$ 0.4	11.7 $\pm$ 0.5	18.0 $\pm$ 0.4	10.6 $\pm$ 0.5	160 $\pm$ 5.8
CI-6	38.1	120.1	113	23.9 $\pm$ 0.2	16.5 $\pm$ 0.3	21.3 $\pm$ 0.2	13.9 $\pm$ 0.3	243 $\pm$ 7.4
CII-1	9.97	29.8	33.9			12.1 $\pm$ 0.2	4.7 $\pm$ 0.3	82 $\pm$ 2.1
CII-2	14.95	45.3	54.1			15.4 $\pm$ 0.4	8.0 $\pm$ 0.4	128 $\pm$ 3.6
CII-3	19.93	56.1	70.1			16.9 $\pm$ 0.3	9.5 $\pm$ 0.3	120 $\pm$ 4.3
CII-4	24.90	68.4	83.7	20.6 $\pm$ 0.4	13.2 $\pm$ 0.5	21.0 $\pm$ 0.3	13.6 $\pm$ 0.3	165 $\pm$ 8.2
CII-5	29.83	79.8	96.7	24.0 $\pm$ 0.4	16.6 $\pm$ 0.4	23.8 $\pm$ 0.2	16.4 $\pm$ 0.3	193 $\pm$ 8.6
CII-6	38.25	116.7	133.3	29.0 $\pm$ 0.4	21.6 $\pm$ 0.4	27.9 $\pm$ 1.1	22.5 $\pm$ 0.4	273 $\pm$ 12.1
CIII-1	10	27.9	32.0			29.0 $\pm$ 1.1	21.6 $\pm$ 1.1	142 $\pm$ 13.5
CIII-2	15	41.5	47.5	41.1 $\pm$ 1.5	33.7 $\pm$ 1.5	41.0 $\pm$ 1.3	33.6 $\pm$ 1.3	217 $\pm$ 7.8
CIII-3	20	53.8	61.5	55.0 $\pm$ 1.7	47.6 $\pm$ 1.7	46.6 $\pm$ 2.0	39.2 $\pm$ 2.0	295 $\pm$ 8.6
CIII-4	25	64.4	73.9	61.7 $\pm$ 1.8	54.3 $\pm$ 1.8	59.3 $\pm$ 3.0	51.9 $\pm$ 3.1	397 $\pm$ 11.8
CIII-5	30	82.2	91.5	70.7 $\pm$ 1.5	63.3 $\pm$ 1.5	70.5 $\pm$ 1.8	63.1 $\pm$ 1.8	518 $\pm$ 15.0

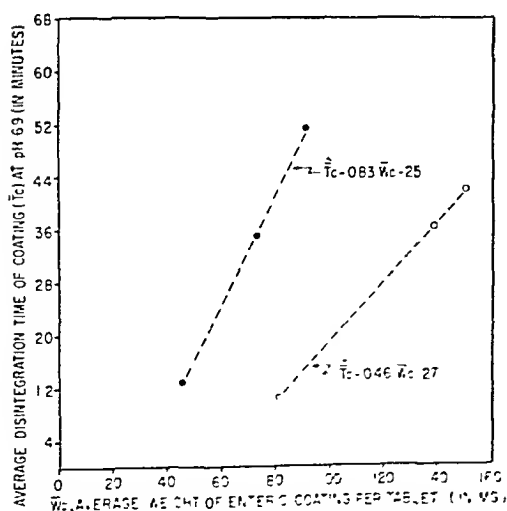
<sup>a</sup> The weight of coating is the difference in weight of 500 enteric coated and 500 subcoated tablets

Fig. 1.—A plot of average disintegration time of the enteric coating in artificial intestinal fluid, pH 6.9 ( $T_c$ , in minutes) against the average weight of enteric coating per tablet ( $W_c$ , in milligrams). Coatings: ● — ● cellulose acetate phthalate, Span 80, propylene glycol, and talc. (Lots BI-1-1, -2, and -3); ○ — ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots BI-1-1, -2, and BI-1-1, -2.)

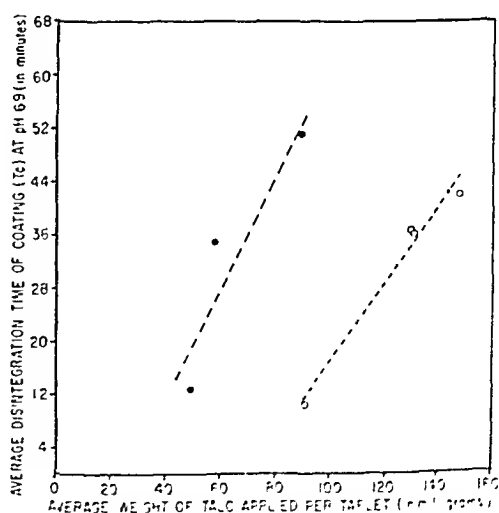


Fig. 2.—A plot of average disintegration time of the enteric coating ( $T_c$ , in minutes) against the average weight of talc applied per tablet (in milligrams). Coatings: ● — ● cellulose acetate phthalate, Span 80, propylene glycol, and talc. (Lots BI-1-1, -2, and -3); ○ — ○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots BI-1, -2, and BI-1-1, -2.)

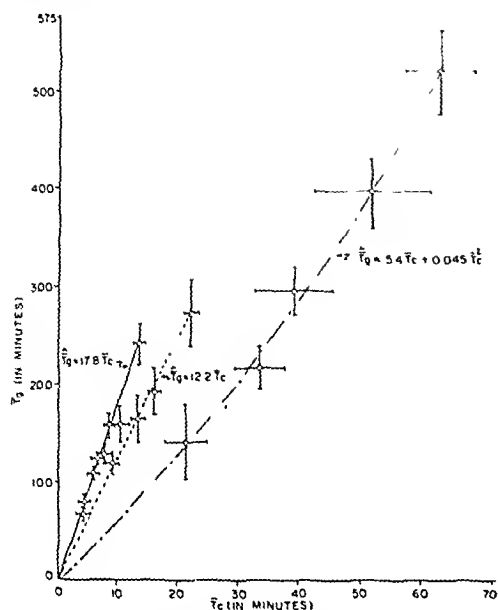


Fig. 3.—A plot of time (in minutes) to the coating attacked end point in simulated gastric fluid U. S. P. ( $T_g$ ) against average disintegration time of the enteric coating ( $T_c$ , in minutes) in artificial intestinal fluid, pH 6.9, when the tablets were placed directly into the latter fluid. Coatings: ○—○ SY-MA copolymer (type 2), dibutyl phthalate, and talc. (Lots CI-1 through -6); ○—○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots CII-1 through -6); ○—○ starch acetate phthalate, dibutyl phthalate, and talc. (Lots CIII-1 through -5.) Vertical and horizontal bars are 99% confidence intervals about the mean ordinate and abscissa values, respectively.

different amounts of talc so that in each case the ratio of talc:enteric polymer varied from lot to lot.

Study C compared coatings prepared from SY-MA copolymer and starch acetate phthalate. In both cases dibutyl phthalate was used as plasticizer. For each enteric polymer, lots of tablets were coated with variable amounts of enteric polymer and talc but the ratio of talc to enteric polymer remained essentially constant from lot to lot.

In order to completely elucidate the relative effects of talc and enteric substance on the disintegration behavior of the resultant coatings it would be necessary to do a fourth type of study in which constant amounts of talc but variable amounts of enteric polymer were applied to a series of lots of tablets. In practice this type of study is the most difficult to perform due to the nature of the pan coating process.

Results of studies A and B indicate that not only is the dusting powder important from the standpoint of the process of enteric coating in the pan and the production of smooth coatings, but also the presence or absence and the amount of dusting powder used with a given enteric polymer largely determines the disintegration behavior of the coatings. Changing the talc:enteric polymer ratio or changing the amounts of dusting powder and polymer per tablet (keeping the ratio constant) changes the disintegration time of the coatings to a different degree for the various coating systems.

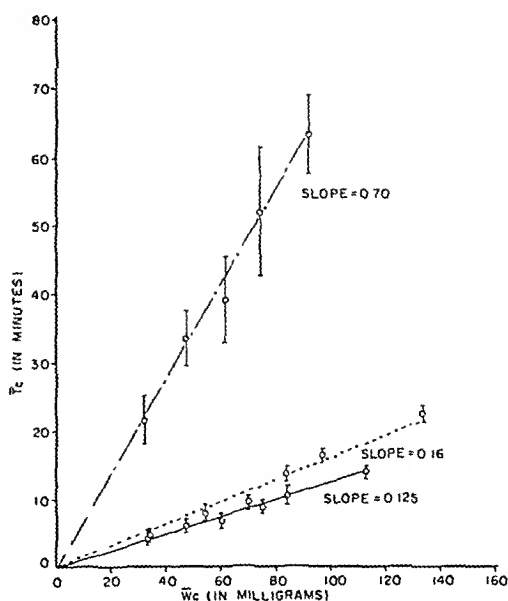


Fig. 4.—A plot of average disintegration time of the enteric coating ( $T_c$ , in minutes) when the tablets were placed directly into artificial intestinal fluid, pH 6.9, against the average weight of enteric coating per tablet ( $W_c$  in milligrams). Coatings: ○—○ starch acetate phthalate, dibutyl phthalate, and talc. (Lots CIII-1 through -5); ○—○ SY-MA copolymer (type 1), dibutyl phthalate, and talc. (Lots CII-1 through -6); ○—○ SY-MA copolymer (type 2), dibutyl phthalate, and talc. (Lots CI-1 through -6.) Vertical bars are 99% confidence intervals about the ordinate values.

Figures 1 and 2 (derived from data of study B, Table IV) indicate that the amount of talc applied per tablet has a marked effect on the disintegration time of both types of coatings in artificial intestinal fluid. From these and similar plots it has been found that the effect of talc on disintegration time seems to be least for SY-MA copolymer coatings, intermediate for starch acetate phthalate coatings, and greatest for cellulose acetate phthalate coatings. However, resistance to simulated gastric fluid is largely determined by the amount of enteric polymer applied per tablet. Incorporation of a minimum amount of the dusting powder into the enteric film usually is essential to get satisfactory acid resistance but an increase in the amount of the dusting powder does not confer increased acid resistance. Because of the variable loss of dusting powder from lot to lot, plots based on the weight of coating, like Fig. 1, usually are more useful for predicting than plots based on weight of powder applied, like Fig. 2. If one assumed that no enteric polymer or plasticizer was lost in the coating pan (or the tablets were assayed for enteric polymer and plasticizer) then a more accurate plot of the type shown in Fig. 2 could be constructed.

Some of the variables involved in study C are plotted in Figs. 3 through 7. These plots are valid only for the ratios of talc:polymer employed.

When interpreting a plot of the type shown as Fig. 6 one must remember that the average talc applied:polymer applied ratio is 3.09 and that the

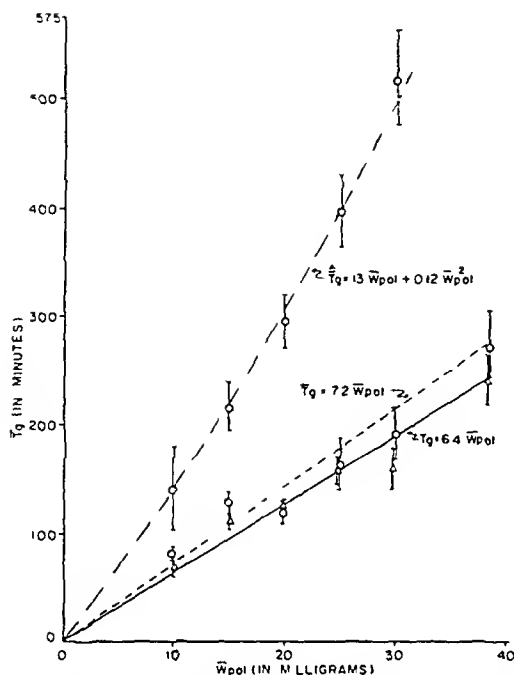


Fig 5—A plot of time (in minutes) to the coating and end point in simulated gastric fluid U S P ( $T_g$ ) against the mean weight of enteric polymer applied per tablet ( $W_{pol}$ , in milligrams). Coatings:  $\bigcirc$  — starch acetate phthalate, dibutyl phthalate, and tale (Lots CIII-1 through 5),  $\bigcirc$  — SY-MA copolymer (type 1), dibutyl phthalate, and tale (Lots CII-1 through 6),  $\triangle$  — SY-MA copolymer (type 2), dibutyl phthalate, and tale (Lots CI-1 through 6). Vertical bars are 99% confidence intervals about the ordinate values.

tale as well as polymer at each level is influencing the intestinal disintegration time of the coating.

The type of plot shown in Fig 3 is an important one for comparison of different enteric coatings. Figure 3 illustrates that the rate of increase of gastric resistance in relation to increase in intestinal disintegration time is greater for the SY-MA copolymer coatings than for the starch acetate phthalate coatings. Comparison of the slopes of the lines at any given  $T_c$  value can be made by differentiating the equation  $\hat{T}_g = 5.4 T_c + 0.045 T_c^2$  for the starch acetate phthalate coatings as follows:

$$\frac{d\hat{T}_g}{dT_c} = 5.4 + 0.09 T_c$$

When  $T_c = 10$  minutes, the instantaneous slope is 6.3 and when  $T_c = 20$  minutes, the slope is 7.2. Over the range studied the slopes for the SY-MA copolymer coatings, namely 12.2 and 17.8, are always greater than the instantaneous slope of the line for the starch acetate phthalate coatings. There is no doubt that the actual values of gastric resistance ( $T_g$ ) for a given weight of enteric polymer applied are higher for both the cellulose acetate phthalate and starch acetate phthalate coatings than for the SY-MA copolymer coatings. This is readily seen in Fig 5. However, it has been shown in dogs (3) that the *in vitro* gastric resistance test is

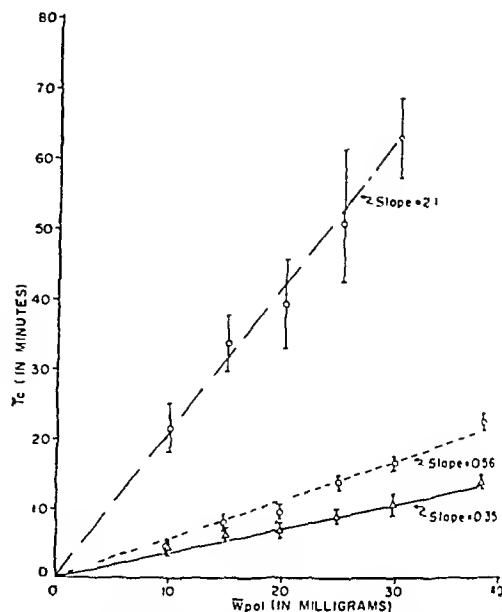


Fig 6—A plot of average disintegration time of enteric coating ( $T_c$ , in minutes) against the average weight of enteric polymer applied per tablet ( $W_{pol}$ , in milligrams). Coatings:  $\bigcirc$  — starch acetate phthalate, dibutyl phthalate, and tale (Lots CIII-1 through 5),  $\bigcirc$  — SY-MA copolymer (type 1), dibutyl phthalate, and tale (Lots CII-1 through 6),  $\triangle$  — SY-MA copolymer (type 2), dibutyl phthalate, and tale (Lots CI-1 through 6). Vertical bars are 99% confidence intervals about the ordinate values.

not as reliable an index of *in vivo* resistance to stomach conditions as the *in vivo* intestinal disintegration test is an index to *in vivo* intestinal disintegration time. Tablets which withstand simulated gastric fluid for from one to four hours may exhibit much greater resistance to stomach conditions in the dog. Two other factors are: (a) it is only necessary to protect the enteric coated dosage form in the stomach for the duration of time that it may be expected to remain in the stomach, and (b) with many drugs it is more important to ensure that the enteric coated dosage form will liberate the contained drug in the intestines than it is to ensure that it will remain intact in the stomach for many hours.

Figures 1, 4, and 6 illustrate that SY-MA copolymer coatings exhibit the lowest dependence of disintegration time in artificial intestinal fluid on weight of enteric coating or weight of enteric polymer applied when compared to starch acetate phthalate or cellulose acetate phthalate coatings. This is extremely important from a manufacturing viewpoint. If the tablet enterer inadvertently applies more than the usual amount of enteric coating the effect on intestinal disintegration time will be much less for SY-MA copolymer coatings than for either starch acetate phthalate or cellulose acetate phthalate coatings.

There has been speculation during the coating of tablets punched with a standard oval punch concerning which part of the coating build up

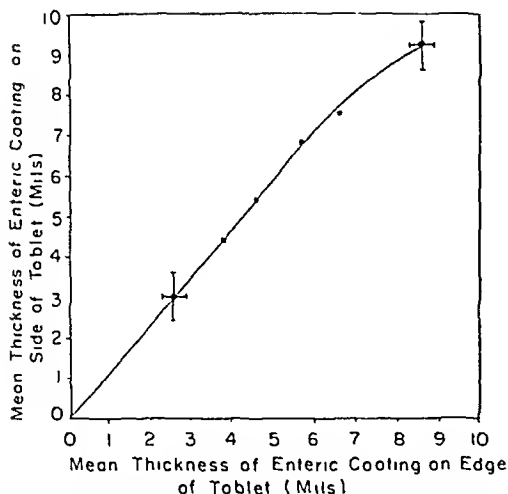


Fig 7.—A plot of average thickness of enteric coating on the side of the tablet (in mils) against average thickness of enteric coating on the edge of the tablet (in mils). Tablets coated with SY-MA copolymer (type 2), dibutyl phthalate, and talc (Lots CI-1 through -6.) Vertical and horizontal bars are 99% confidence intervals about the mean ordinate and abscissa values, respectively.

faster. Figure 7 shows a plot of average thickness of enteric coating on the side of the tablet (i.e., dimension of thickness of the tablet) against average thickness of enteric coating on the edge of the tablet (i.e., dimension of diameter of the tablet). This curve has a sigmoidlike shape, at first curving slightly upwards then reaching an inflection point, then curving downwards. Hence in this case, the coating on the side of the tablet built up faster than coating on the edge at first; then a point was reached where the build-up was equal for both dimensions;

and finally, the coating on the edge built up faster than the coating on the side of the tablet.

## SUMMARY AND CONCLUSIONS

1. The effect of some of the variables in the enteric coating process on the disintegration behavior of three modern enteric coatings was elucidated.

2. Some of the properties of a new improved enteric coating were reported. The new coating is prepared from styrene-maleic acid copolymer, dibutyl phthalate, and talc.

3. Certain empirical relationships which are useful in predicting the properties of enteric coatings and in comparing one type of coating with another were presented.

4. The concentration levels of talc and enteric polymer were both shown to be very important in determining the disintegration characteristics of three enteric coatings.

## REFERENCES

- (1) Bauer, C. W., and Geraughty, R. J., *THIS JOURNAL, Pract. Pharm. Ed.*, 14, 504 (1953).
- (2) Hawkins, D. B., and Thompson, H. O., *THIS JOURNAL*, 42, 424 (1953).
- (3) Wagner, J. G., Veldkamp, W., and Long, S., *ibid.*, 47, 681 (1958).
- (4) Wagner, J. G., Brignall, T. W., and Long, S., *ibid.*, 48, 244 (1959).
- (5) Wagner, J. G. (to The Upjohn Co.), U. S. pat. 2,897,121, July 28, 1959.
- (6) Abbott Laboratories, British pat. 760,403, October 31, 1956.
- (7) Tauch, E. J. (to E. I. du Pont de Nemours & Co.), U. S. pat. 2,400,489, December 6, 1949.
- (8) Garrett, E. R., and Guile, R. L., *J. Am. Chem. Soc.*, 73, 4533 (1951).
- (9) Barrett, G. R. (to Monsanto Chemical Co.), U. S. pat. 2,675,320, April 13, 1954.

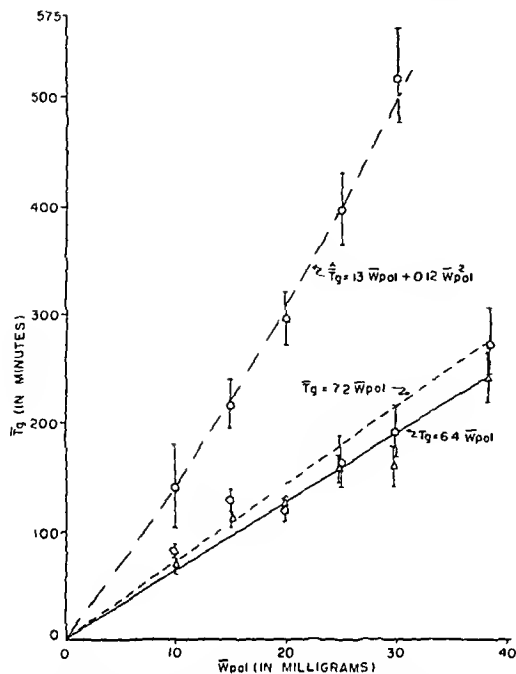


Fig 5 — A plot of time (in minutes) to the coating and end point in simulated gastric fluid U S P ( $T_g$ ) against the mean weight of enteric polymer applied per tablet ( $\bar{W}_{pol}$ , in milligrams). Coatings: ○ — starch acetate phthalate, dibutyl phthalate, and talc (Lots CIII-1 through -5), ○ — SY-MA copolymer (type 1), dibutyl phthalate, and talc (Lots CII-1 through -6), △ — SY-MA copolymer (type 2), dibutyl phthalate, and talc (Lots CI-1 through -6). Vertical bars are 99% confidence intervals about the ordinate values.

talc as well as polymer at each level is influencing the intestinal disintegration time of the coating.

The type of plot shown in Fig 3 is an important one for comparison of different enteric coatings. Figure 3 illustrates that the rate of increase of gastric resistance in relation to increase in intestinal disintegration time is greater for the SY-MA copolymer coatings than for the starch acetate phthalate coatings. Comparison of the slopes of the lines at any given  $T_c$  value can be made by differentiating the equation  $\hat{T}_g = 5.4 T_c + 0.045 T_c^2$  for the starch acetate phthalate coatings as follows:

$$\frac{d\hat{T}_g}{dT_c} = 5.4 + 0.09 T_c$$

When  $T_c = 10$  minutes, the instantaneous slope is 6.3 and when  $T_c = 20$  minutes, the slope is 7.2. Over the range studied the slopes for the SY-MA copolymer coatings, namely 12.2 and 17.8 are always greater than the instantaneous slope of the line for the starch acetate phthalate coatings. There is no doubt that the actual values of gastric resistance ( $T_g$ ) for a given weight of enteric polymer applied are higher for both the cellulose acetate phthalate and starch acetate phthalate coatings than for the SY-MA copolymer coatings. This is readily seen in Fig 5. However, it has been shown in dogs (3) that the *in vitro* gastric resistance test is

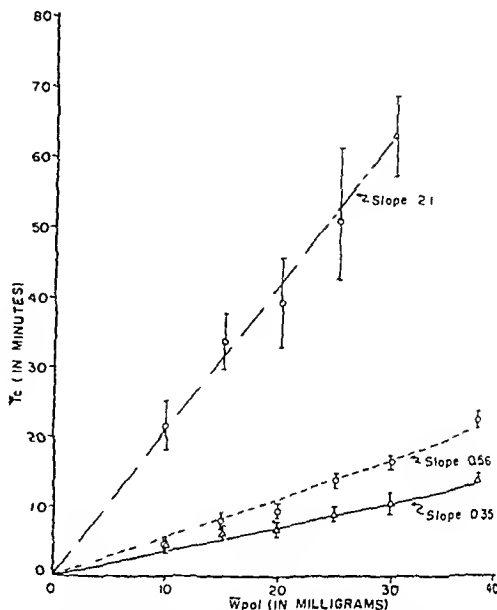


Fig 6 — A plot of average disintegration time of enteric coating ( $T_c$ , in minutes) when the tablets were placed directly into artificial intestinal fluid pH 6.9, against the average weight of enteric polymer applied per tablet ( $\bar{W}_{pol}$ , in milligrams). Coatings: ○ — starch acetate phthalate, dibutyl phthalate, and talc (Lots CIII-1 through -5), ○ — SY-MA copolymer (type 1), dibutyl phthalate, and talc (Lots CII-1 through -6), △ — SY-MA copolymer (type 2), dibutyl phthalate, and talc (Lots CI-1 through -6). Vertical bars are 99% confidence intervals about the ordinate values.

not as reliable an index of *in vivo* resistance to stomach conditions as the *in vitro* intestinal disintegration test is an index to *in vivo* intestinal disintegration time. Tablets which withstand simulated gastric fluid for from one to four hours may exhibit much greater resistance to stomach conditions in the dog. Two other factors are: (a) it is only necessary to protect the enteric coated dosage form in the stomach for the duration of time that it may be expected to remain in the stomach, and (b) with many drugs it is more important to ensure that the enteric coated dosage form will liberate the contained drug in the intestines than it is to ensure that it will remain intact in the stomach for many hours.

Figures 1, 4, and 6 illustrate that SY-MA copolymer coatings exhibit the lowest dependence of disintegration time in artificial intestinal fluid on weight of enteric coating or weight of enteric polymer applied when compared to starch acetate phthalate or cellulose acetate phthalate coatings. This is extremely important from a manufacturing viewpoint. If the tablet coater inadvertently applies more than the usual amount of enteric coating the effect on intestinal disintegration time will be much less for SY-MA copolymer coatings than for either starch acetate phthalate or cellulose acetate phthalate coatings.

There has been speculation during the coating of tablets punched with a standard oval punch concerning which part of the coating builds up

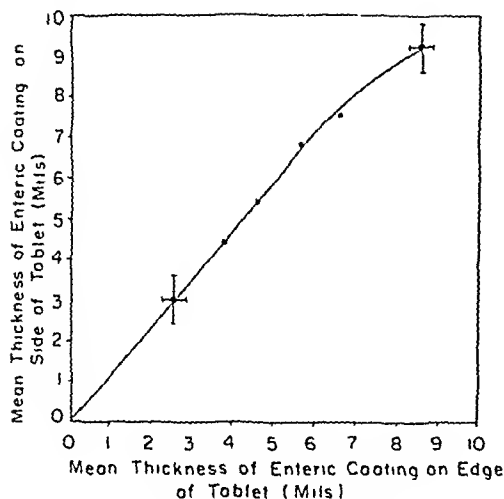


Fig 7.—A plot of average thickness of enteric coating on the side of the tablet (in mils) against average thickness of enteric coating on the edge of the tablet (in mils). Tablets coated with SY-MA copolymer (type 2), dibutyl phthalate, and talc (Lots CI-1 through -6). Vertical and horizontal bars are 99% confidence intervals about the mean ordinate and abscissa values, respectively.

faster. Figure 7 shows a plot of average thickness of enteric coating on the side of the tablet (i.e., dimension of thickness of the tablet) against average thickness of enteric coating on the edge of the tablet (i.e., dimension of diameter of the tablet). This curve has a sigmoidlike shape, at first curving slightly upwards then reaching an inflection point, then curving downwards. Hence in this case, the coating on the side of the tablet built up faster than coating on the edge at first, then a point was reached where the build-up was equal for both dimensions,

and finally, the coating on the edge built up faster than the coating on the side of the tablet.

## SUMMARY AND CONCLUSIONS

1 The effect of some of the variables in the enteric coating process on the disintegration behavior of three modern enteric coatings was elucidated.

2 Some of the properties of a new improved enteric coating were reported. The new coating is prepared from styrene-maleic acid copolymer, dibutyl phthalate, and talc.

3 Certain empirical relationships which are useful in predicting the properties of enteric coatings and in comparing one type of coating with another were presented.

4 The concentration levels of talc and enteric polymer were both shown to be very important in determining the disintegration characteristics of three enteric coatings.

## REFERENCES

- (1) Bauer, C. W., and Geraughty, R. J., *THIS JOURNAL, Pract Pharm Ed.*, 14, 504 (1953).
- (2) Hawkins, D. B., and Thompson, H. O., *THIS JOURNAL*, 42, 424 (1953).
- (3) Wagner, J. G., Veldkamp, W., and Long, S., *ibid.*, 47, 681 (1958).
- (4) Wagner, J. G., Brignall, T. W., and Long, S., *ibid.*, 48, 244 (1959).
- (5) Wagner, J. G. (to The Upjohn Co.), U. S. pat. 2,897,121, July 28, 1959.
- (6) Abbott Laboratories British pat. 760,403, October 31, 1956.
- (7) Tauch, E. J. (to E. I. du Pont de Nemours & Co.), U. S. pat. 2,490,489, December 6, 1949.
- (8) Garrett, E. R., and Guile, R. L., *J. Am. Chem. Soc.*, 73, 4533 (1951).
- (9) Barrett, G. R. (to Monsanto Chemical Co.), U. S. pat. 2,675,320 April 13, 1954.



# Enteric Coatings IV\*

## *In Vivo* Testing of Granules and Tablets Coated With Styrene-Maleic Acid Copolymer

By JOHN G. WAGNER, WILLIAM VELDKAMP, and STUART LONG

Preliminary tests in human subjects have indicated that the period of time which tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc resist the stomach contents of human subjects appears to be well predicted by an *in vitro* test in simulated gastric fluid U. S. P. Both freshly coated granules and stability samples of tablets coated with this formulation disintegrated rapidly in the small intestine of the dogs and human subjects, respectively. The site of disintegration was the duodenum or jejunum where optimal absorption of many therapeutic agents would be expected.

IN 1933, LOZINSKI AND DIVER (1) introduced the use of roentgenoscopy with barium sulfate tablets and the barium meal to follow the path of enteric coated tablets through the stomach and into the intestine. A year later Bukey and Brew (2) introduced reontgenography to follow the path of enteric coated dosage forms through the gastrointestinal tract of human subjects. Roentgenoscopy and roentgenography have become accepted as classical methods to study enteric coated dosage forms and many investigators have reported such studies during the past quarter century.

One of the difficulties with the methods was pointed out by Garland (3) who noted that there could be a great difference between disintegration and absorption depending on what part of the intestine the dosage form had reached when it disintegrated. It is evident that many drugs are absorbed faster and more efficiently in the proximal parts of the small intestine. Convreur, *et al.* (4), stated that an excellent enteric coating should logically be disintegrated in the first part of the small intestine in order to allow optimum absorption of the mass medication. Hence, an enteric coated dosage form which protects the drug in the stomach but releases the drug rapidly once the dosage form enters the small intestine would be optimal for these drugs.

Some enteric coated tablets and capsules do not disintegrate very rapidly in the small intestine while others have been reported to disintegrate quite rapidly. We have calculated from the data of Crane and Wruble (5) that 174 tablets freshly coated with ammoniated shellac were emptied from the stomachs of human subjects after an average time of 3.61 hours (standard

deviation 1.47 hours) and disintegrated in the small intestine after an average time of 2.55 hour (standard deviation 1.08 hours). One lot of tablets coated with ammoniated shellac was tested initially and after storage for one year at room temperature. None of the freshly coated tablets remained intact in the small intestine whereas 7 out of 35, or 20 per cent, of the one year stability sample did remain intact in the small intestine. Similarly, Tarnowski (6) reported that shellac coated PAS granules progressively change in *in vitro* and *in vivo* disintegration behavior releasing drug much slower as they age at room temperature, and Hinkle (7) reported that radiologists observe many intact enteric coated dosage forms in the distal parts of the intestinal tract. The only conclusion with respect to *intestinal disintegration time* that can be drawn from the studies of Hodge, Forsyth, and Ramsey (8) is that most of the tablets and capsules coated with cellulose acetate phthalate, which they tested, apparently disintegrated from within a few minutes to up to four hours after the coated dosage forms left the stomach and entered the intestines of human patients. Convreur, *et al.* (4), reported that in five human subjects tablets containing barium sulfate and radioactive iodide and coated with cellulose acetate phthalate-diethyl phthalate emptied from the stomach after an average time of 2.63 hours (range 1.5 to 4.3 hours) and disintegrated in the intestines within ten to twenty minutes after passing through the pylorus. The same authors reported results obtained in two other patients in whom different tablet cores but with the same coating were tested. They reported that in these patients the resistance of the coating was too high and that the tablets were probably in the cecum or descending end of the sigmoid before the coating ruptured. Other investigators (9, 10) pointed out that certain enteric coatings

\* Received August 13, 1959, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

The authors wish to express their gratitude to Dr. J. C. Volderauer who exposed and interpreted the roentgenograms in the clinical trials and to Dr. J. P. Webb who arranged the clinical testing.

containing cellulose acetate phthalate allow release of the medicament in the stomach and will not pass the accepted test for enteric coatings particularly after aging.

After testing many of the known enteric coatings we became acutely aware that they all suffered from basic deficiencies. The previous communication (11) described an improved enteric coating prepared from styrene-maleic acid copolymer, dibutyl phthalate, and talc. Preliminary *in vivo* testing of granules and tablets coated with this formulation by the roentgenographic method are described in this report.

## EXPERIMENTAL

**Enteric Coated Tablets.**—In the clinical studies with human subjects three different stability samples of enteric coated barium sulfate tablets were tested. The composition of these lots, CI-3, CI-6, and BI-1, have been described previously (11). Two of the lots, CI-3 and CI-6, were stored for five months at room temperature and one lot, BI-1, was stored for twelve months at 40° then for four months at room temperature before administration to the subjects.

**Protective Coated Granules.**—Pill starters containing 50% by weight of barium sulfate were prepared by applying a mixture of barium sulfate U. S. P., talc, starch, and cane sugar to sugar crystals in a pill tub using a water spray. In both simulated gastric fluid U. S. P., pH 1.2, and simulated intestinal fluid U. S. P., pH 7.5, the unprotected pill starters were completely disintegrated to a fine powder within one minute. The protective coating solution was prepared from 127.5 Gm. of styrene-maleic acid copolymer, 15.3 Gm. of di-*n*-butyl phthalate, 850 ml. of S. D. alcohol 3A, anhydrous, and 25.5 ml. of mineral oil U. S. P. In the preparation of Lot 1 an amount of 566.5 ml. of the above solution was applied in 22 aliquots to 2,300 Gm. (approx. 1.75 million granules) of the pill starters using 350 Gm. of a mixture of 80 parts by weight U. S. P. supreme talc and 20 parts by weight of magnesium stearate as a dusting powder. One-half of these coated granules were coated further with 309 ml. of the above solution, applied in 23 aliquots, using 150 Gm. of the same powder mixture as a dusting powder to produce Lot 2. Each lot of coated granules was dried for seventy-two hours at 105° F. The physical properties of the coated granules are shown in Table I.

TABLE I.—PHYSICAL PROPERTIES OF COATED GRANULES

	Lot 1	Lot 2
Average diameter, mils	44.0	46.3
Average thickness of protective coating, mils	1.6	2.75
Average weight of protective coating, mg./granule	0.25	0.44
Average weight of SY-MA copolymer applied, mg./granule	0.047	0.099

Since quantitative determination of the barium sulfate liberated from such a coated product would present great experimental difficulties disintegration tests carried out on these granules in various buffers utilized only a visual end point. In each test one hundred granules were enclosed in a glass tube which was fitted with a No. 20 mesh screen at each end. The tube was suspended from the arm of the U. S. P. disintegration apparatus and allowed to move through 100 ml. of buffer at 37°. Results obtained on Lot 2 are summarized in Table II.

**Clinical Testing of Enteric Coated Barium Sulfate Tablets by X-ray Techniques.**—Three adult, healthy male human subjects were administered two or six of the enteric coated barium sulfate tablets with a glassful of water. In all tests except one (J. G. W. with lot BI-1) the subjects ate a normal lunch, had a cup of coffee at 3:00 p. m., swallowed the tablets followed by a glassful of water at 5:00 p. m., then ate a light but normal supper at 6:00 p. m. When J. G. W. was administered lot BI-1 the subject ate a normal lunch at 12:30 p. m. and had a cup of coffee at 3:00 p. m. Two tablets were taken at 5:00 p. m., two tablets at 6:00 p. m., and two tablets at 6:30 p. m., in each case with a half glassful of water. In each test the first X-ray film was exposed at 7:00 p. m.  $\pm$  five minutes. The tablets were then followed thereafter by means of the fluoroscope and X-ray films. The details of the food eaten by the subjects and the interpretations of the individual films made by Dr. J. C. Volderauer, the radiologist, are too lengthy to be reported in detail here. Sixteen of the tablets remained in the stomachs of the subjects during the observation period. Table III summarizes results obtained with these tablets. Twelve of the tablets emptied from the stomach and disintegrated in the small intestine during the observation period. Table IV summarizes the results obtained with the latter tablets. It is of interest to note that when tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc disintegrate in the small intestine they disintegrate very rapidly and completely, similar to their *in vitro* behavior in simulated intestinal fluid, pH 6.9.

**Testing the Protective Coated Granules in the Dog by X-Ray Techniques.**—The first two experiments tested the disintegration rate of the unprotected pill starters in the stomachs of dogs. A pink No. 3 capsule containing approximately 300 unprotected pill starters was administered to a dog which had been starved for twenty-four hours. A roentgenogram taken one and three-quarters of a minute later disclosed the intact capsule in the stomach of the dog. A roentgenogram taken five minutes after the first one disclosed that the capsule had broken and that all of the granules had disintegrated. A third roentgenogram taken five minutes later showed the barium sulfate well dispersed.

A pink No. 3 capsule containing approximately 310 unprotected pill starters was administered later the same day to the same dog. A roentgenogram taken one minute later showed the gelatin capsule in the process of breaking. A roentgenogram taken two and one half minutes after administration showed that the capsule had broken and that the granules had disintegrated. Additional plates taken four



TABLE IV.—COMPARISON OF RESULTS OBTAINED IN MAN, WHERE TABLETS ENTERED THE SMALL INTESTINE DURING THE OBSERVATION PERIOD, TO *In Vitro* TEST IN ARTIFICIAL INTESTINAL FLUID, pH 6.9

Tablet Lot	Subject	No. of Tablets	Time from Administration, min.	Interpretation of Roentgenogram	<i>In Vitro</i> Test Average $\pm$ S. E., min. <sup>a</sup>
CI-6. After storage for five months at room temp.	D. H. G.	1	120 147 and subsequent films	Tablets intact in lower jejunum or upper ileum No trace of tablets, assumed to be completely disintegrated	30 $\pm$ 0.7
CI-3. After storage for five months at room temp.	J. G. W.	4	123	No trace of tablets, all four tablets completely disintegrated	16 $\pm$ 0.3
	D. H. G.	1	121 and subsequent films	No trace of tablets, completely disintegrated	
BI-1. After storage for twelve months at 40°, then for four months at room temp.	J. G. W.	6	30 <sup>b</sup>	One tablet in first part of duodenum and five tablets in about middle of jejunum	31 $\pm$ 1.6
			30 to 45	Fluoroscopic examination indicated no change	
			45	One tablet still in duodenum, five tablets in jejunum and at least two of these tablets had an uneven surface indicative they had started to disintegrate	
			54	One tablet had moved from the duodenum to first part of the jejunum and showed signs of disintegrating; of the other five tablets, one was disintegrated into a number of fragments and the other four showed earlier signs of disintegration	
			63	Fluoroscopic examination indicated little change	
			65	One tablet in upper jejunum showed more evidence of disintegration since the last film; of the other five tablets one was completely disintegrated	
			80	One tablet in many fragments and fragments lower in jejunum than in previous film; other five tablets had moved further down in small bowel and definitely disintegrated	
			90	All tablets completely disintegrated and homogenous bolus of barium sulfate visible	

<sup>a</sup> Average  $\pm$  the standard error of the average disintegration time in artificial intestinal fluid, pH 6.9. The end point was taken when 99 to 100% of the entire tablet was through the screen of the U. S. P. disintegration apparatus.

<sup>b</sup> Thirty minutes after administration of the last two of six tablets. Two other tablets had been administered sixty minutes before this film and the remaining two tablets ninety minutes before this film.

to stomach contents of the dog for at least eight hours and were about 95% intact after six hours in simulated gastric fluid. The correlation observed with the enteric coated tablets in the dogs (12) was not nearly as good. In the dogs the *in vivo* resistance to stomach contents was considerably greater than the *in vitro* resistance to simulated gastric fluid.

All of the enteric coated tablets tested which emptied from the stomachs of the human subjects during the observation period disintegrated completely high up in the small bowel. Even the lot which had been stored for twelve months at 40° followed by four months at room temperature disintegrated completely in the upper small bowel.

These tablets were attacked in the jejunum, and although no direct correlation could be made, the disintegration time in artificial intestinal fluid, pH 6.9, appeared to be a good indication of the disintegration time *in vivo*, measured from the time the tablets emptied from the stomach. In interpreting Table IV one must remember the stomach retention time of the tablets is also involved. For example, in the test of lot BI-1 in subject J. G. W. one tablet disintegrated completely twenty-four minutes after it was first observed in the small intestine and the other five tablets completely disintegrated fifty minutes after they were first observed in the small intestine.

The phenomenon of slow stomach emptying of

TABLE V—INTERPRETATION OF ROENTGENOGRAMS OBTAINED IN THE FOURTH EXPERIMENT WITH THE DOG

Time from Administration, min.	Interpretation of Roentgenogram
1 55	Intact capsule just entered the stomach
14 8	Capsule broken; coated granules clustered in stomach
30 1	Granules dispersed in the stomach
45, 60, 75, 90, 105, 120, 135	No apparent change from the above
150	Barium sulfate appeared in first loop of duodenum
165	Barium sulfate further down duodenum but less intense in first part of duodenum indicative that disintegration of some granules had occurred since the last film
180, 210	Barium sulfate very faint in duodenum. Spot in stomach less intense than formerly
240	Barium sulfate spreading throughout small bowel
270	More granules in duodenum
300	Barium sulfate dispersed and less intense in duodenum indicating disintegration had occurred since last film
330	Barium sulfate well distributed throughout bowel. Large cluster of granules still in the stomach
420	Small streak of barium sulfate again seen in duodenum
480	More barium sulfate in duodenum compared with last film taken at 390 minutes
Test terminated	Stomach spot less intense
	Barium sulfate dispersed throughout small bowel
	Cluster of coated granules still in the stomach but becoming much smaller

coated granules which we observed in the dog is not necessarily restricted to granules nor to dogs. It was reported about sixty years ago that stomach emptying of human beings is a first order process (13). The rate constants or half-times for the process appear to depend upon the nature of the material introduced into the stomach and in some cases it is several hours before a given test meal is emptied. This is evidence that possibly one of the factors involved in the overall sustained effect achieved with certain coated granule preparations is the stomach-emptying rate. The slow stomach-emptying of our coated granules and rapid disintegration of the small clusters as they are released into the duodenum indicate there may be even another possible mechanism of attaining sustained action of a drug. We are merely presenting this as a hypothesis. Our other publications (14, 15) show that sustained plasma 17-hydroxycorticosteroid levels were achieved in human subjects who were

administered granules containing prednisolone and coated with the entire formulation discussed in this paper.

## SUMMARY AND CONCLUSIONS

Preliminary testing of granules and stability samples of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc have indicated the following:

1 The period of time which the coated tablets resist the stomach contents of human subjects appears to be well predicted by an *in vitro* test in simulated gastric fluid, pH 1.2.

2 The coated granules resisted the stomach contents of the starved dog for at least an eight-hour period and the granules were progressively emptied from the stomach of the dog over a period of at least eight hours.

3. All of the enteric coated granules which emptied from the stomach of the dog and all of the enteric coated tablets which emptied from the stomachs of the human subjects during the observation periods disintegrated completely in the proximal part of the small bowel. This was true even for one lot of coated tablets which had been stored for twelve months at 40° and then for four months at room temperature.

4 The hypothesis is presented that slow stomach emptying of small coated granules followed by fairly rapid dissolution of small clusters of the granules as they enter the upper small intestine may be an effective method of attaining prolonged action of certain therapeutic agents.

## REFERENCES

- (1) Lozinski, E., and Diver, G. R., *THIS JOURNAL*, 22, 143 (1933).
- (2) Bukey, F. S., and Brew, M., *ibid*, 23, 1217 (1934).
- (3) Editorial, *New Engl J Med*, 254, 963 (1956).
- (4) Convreur, A., and Collaborators, Partial Translation of "Les Enrobages Modernes Des Dragées et Des Pilules," translated and distributed by Distillation Products Industries, Division of Eastman Kodak Co., Rochester 3, N. Y.
- (5) Crane, A. W., and Wruble, M., *Am J Roentgenol Radium Therapy*, 39, 450 (1938).
- (6) Tarnowski, C. E., *Am Rev Tuberc Pulmonary Diseases*, 76, 159 (1957).
- (7) Hinkle, C. L., *Am J Roentgenol Radium Therapy*, 65, 575 (1951).
- (8) Hodge, H. C., Forsyth, H. H., and Ramsey, G. H., *J Pharmacol Exptl Therap*, 80, 241 (1944).
- (9) Clymer, H. A., and Donnell, M., (to Smith Kline & French Laboratories) U. S. pat. 2,540,970, February 6, 1951.
- (10) Abbott Laboratories, British pat. specification 760,403, October 31, 1956.
- (11) Wagner, J. G., and Long, S., *THIS JOURNAL*, 48, 121 (1956).
- (12) Wagner, J. G., Veldkamp, W., and Long, S., *ibid*, 47, 681 (1955).
- (13) Hunt, J. N., *Physiol Revs*, 39, 491 (1959).
- (14) Wagner, J. G. (to the Upjohn Co.), U. S. pat. 2,897,121, July 28, 1959.
- (15) Wagner, J. G., Carpenter, O. S., and Collins, E. J., *J Pharmacol Exptl Therap*, in press.

# Enteric Coatings V\*

## pH Dependence and Stability

By JOHN G. WAGNER, GEORGE W. RYAN, ERNEST KUBIAK,  
and STUART LONG

Evidence is presented that the terms "stable in acid solutions" and "soluble in alkaline solutions" should not be applied to enteric coatings. The enteric substances studied dissolve in buffers of pH less than 7 and tablets coated with these substances disintegrate in fluids having a pH less than 7. It was demonstrated that the critical pH range in which tablets coated with styrene-maleic acid copolymer show a progressive decrease in disintegration time corresponds to a narrow pH range on either side of the  $pK_1$  of the copolymer. It is in this region that the greatest change in per cent ionization of the copolymer occurs. Relating these *in vitro* data with *in vivo* data, formerly reported, will require adjustment of current concepts of enteric coatings. It was shown that the disintegration times of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc in artificial intestinal fluid, pH 6.9, changed very little after storage of the coated tablets for prolonged periods at 4°, room temperature, 40°, and 47°. Tablets coated with the other enteric compositions exhibited either decreased resistance to simulated gastric fluid or markedly increased disintegration times in artificial intestinal fluids after prolonged storage. It was demonstrated that the average disintegration time observed after storage of enteric coated tablets for one month at 47° yields an excellent prediction of the average disintegration time of the tablets after storage for two years at room temperature.

THIS COMMUNICATION has two principal purposes. First, it will be shown that the new enteric coating, formerly discussed (1), disintegrates *in vitro* at pH values very much lower than reported for any other coating. Secondly, the properties of this coating will be compared to those of other enteric coatings, both initially and after aging under various conditions.

The concept that an enteric coating is one which is "stable in acid solutions" but "soluble in alkaline solutions" should be completely forgotten. The basic definition of an enteric coating should only be in terms of resistance to stomach contents and disintegration in intestinal contents. *In vitro* tests are essential for quality control, research, and stability testing. However, we do not believe that *in vivo* results can be predicted from *in vitro* tests unless a valid correlation is established first.

During the past quarter century the *in vitro*-*in vivo* correlations pertaining to enteric coatings have led to a trend to find coatings which disintegrate at more and more acidic pH values. Wruble (2) reported that below pH 6.4, ammoniated shellac coatings were quite resistant but at or above this pH the coatings showed definite signs of attack. Malm, *et al.* (3), showed that a cellulose acetate phthalate, containing 10 per cent carboxyl, dissolved at pH 5.7 to 5.9 under their test conditions. Wagner, *et al.* (4), showed that a starch acetate phthalate, containing 9.2 per cent carboxyl dissolved at pH

5.55 under the same test conditions. In this paper evidence is presented that a coating, containing styrene-maleic acid copolymer, disintegrates *in vitro* at a pH as low as 3.7. It was formerly shown (5), however, that tablets and granules bearing such a coating possessed good enteric properties in the dog and man. The advantage of susceptibility to a lower (more acidic) pH is that disintegration of the coated dosage forms will occur in the proximal parts of the intestinal tract where maximal absorption of many drugs and foodstuffs occur.

Few published studies of enteric coatings have included data concerning the stability of the coatings. Scoville (6), Bukey and Rhodes (7), and Cooper (8) discussed the stability of formalized-gelatin coatings. Sampson, *et al.* (9), reported that one lot of enteric coated quinidine sulfate tablets gave imperfect dissolution after six months storage, while another lot, after similar storage, gave results essentially the same as those obtained with freshly prepared tablets. Four weeks' storage at room temperature and 0° storage were utilized by Bauer and Geraughty (10) and Stoklosa and Ohmart (11) to evaluate their enteric coatings. A patent (12) discussed the use of low pH powders with cellulose acetate phthalate to improve the stability of the coatings. In this communication the stability of many different enteric coatings after prolonged periods of storage at 4°, room temperature, 40°, and 47° is reported.

The criteria, by which the stability of the coatings were measured, were resistance to

\* Received August 13, 1959, from the Research Laboratories of the Upjohn Co., Kalamazoo, Mich

simulated gastric fluid U. S. P. and disintegration time in artificial intestinal fluids, pH 6.9 and 7.5.

EXPERIMENTAL

**Materials.**—The styrene-maleic acid copolymer, starch acetate phthalate, cellulose acetate phthalate, dibutyl phthalate, and talc have been described previously (1, 4). Sorbitan monooleate used was Span 80 (Atlas Powder Co.).

Resin SC-2 was obtained from Monsanto Chemical Co. This resin contains styrene and maleic anhydride residues but is not a straight styrene-maleic acid copolymer. The resin was hydrolyzed by the method of Ferry, *et al.* (13). The hydrolyzed Resin SC-2 was dried for three days at 101°F and 10% relative humidity.

The compositions of the various artificial gastric and intestinal fluids used in this study are shown in Table I.

**Enteric Coated Tablets.**—Lots BI-1, BI-2, BII-1, BII-2, CII-4, CII-5, and CII-6, coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc, have been described previously (1). Lot N was coated with the same mixture of ingredients. Lots D through I were coated with ammoniated shellac as described in the patent of Wruble (14). Lot T was comprised of tablets of lot BII-1 which were film-coated with carboxymethylcellulose and talc after storing for one hundred and fifty-five days at room temperature. Lot U was comprised of tablets

of lot BI-2 which were film-coated with carboxymethylcellulose and talc after storing for one hundred fifty-seven days at room temperature. The compositions of the remainder of the enteric coatings are shown in Table II. All tablets were coated by conventional pan-coating methods using talc as a dusting powder.

METHODS

**Potentiometric Titrations.**—About 0.3 Gm., accurately weighed, of styrene-maleic acid copolymer or hydrolyzed Resin SC-2 was suspended in 200 ml. of boiling water. The suspension was heated on the steam bath with stirring until a homogeneous solution was obtained. The solution was cooled to room temperature and then titrated with 0.1 N sodium hydroxide. The pH of the solution after the addition of each increment of standard base was determined with a Cambridge pH meter fitted with an electric eye. The pH meter was calibrated with Harleco buffer, pH 7.00, at the temperature of the test solution. The inflection point was determined by plotting  $\Delta \text{pH}/\Delta V$  against  $V$ , where  $V$  was the volume of standard base added.

**Disintegration Tests.**—In all tests, the U. S. P. disintegration apparatus, without plastic disks, was used. The fluids were maintained at  $37 \pm 2^\circ$ . Unless otherwise indicated the end point for disintegration tests in artificial intestinal fluids was taken when 99 to 100% of the entire tablet was

TABLE I—COMPOSITION OF FLUIDS USED IN THE DISINTEGRATION TESTS

Ingredient	Artificial Gastric Fluids		Artificial Intestinal Fluids				
	pH 1.2	pH 3.0	pH 3.7	pH 4.0	pH 4.8	pH 6.9	pH 7.5
Pancreatin 3X U. S. P.	a		3.33 Gm.	3.33 Gm.	3.33 Gm.	3.33 Gm.	b
Ox bile extract U. S. P.	a		4.0 Gm.	4.0 Gm.	4.0 Gm.	4.0 Gm.	b
Pepsin	a	3.2 Gm.					b
0.2 M Potassium biphosphate	a					250.0 ml.	b
0.2 M Potassium biphthalate	a		250.0 ml.	250.0 ml.	250.0 ml.		b
0.1 N Hydrochloric acid	a	204.0 ml.	49.75 ml.				b
0.2 M Sodium hydroxide	a			2.0 ml.	118.25 ml.	140.0 ml.	b
Deionized water, q. s.	a	1,000 ml.	1,000 ml.	1,000 ml.	1,000 ml.	1,000 ml.	b
a Simulated gastric fluid U. S. P.			b Simulated intestinal fluid U. S. P.				

TABLE II—COMPOSITION OF ENTERIC COATINGS

Lot of Enteric Coated Tablets	Sub-coated Tablets Used	Amount of Ingredient Applied Per Tablet									
		Styrene-Maleic Acid Copolymer Type I, mg	Hydrolyzed Resin SC-2, mg	Starch Acetate Phthalate, mg	Cellulose Acetate Phthalate, mg	Shellac, mg	Mineral Oil, ml	Dibutyl Phthalate, mg	Sorbitan Monooleate, ml	Propylene Glycol U. S. P., ml	Talc, mg
J	I <sup>a</sup>	25				3					136
K	I	25				3		3			125
L	IV <sup>a</sup>			21			0.0063		0.0021	0.0063	79
M	d			15				1.8			42
N	e	37.5						4.5			234
O	f		17.3					3.5			57
P	e		22.5					4.5			142
Q	I	7.5			7.5		0.005	3			80
R <sup>b</sup>	I	25			12		0.0036	3	0.0012	0.0036	122
S <sup>c</sup>	I	40			12		0.0063	4.8	0.0012	0.0036	198

a See Table II of previous communication (15).  
b A double enteric coated tablet with internal SY MA copolymer coating and external cellulose acetate phthalate coating separated by a carboxymethylcellulose barium sulfate layer.  
c A double enteric coated tablet with internal cellulose acetate phthalate coating and external SY MA copolymer coating separated by a carboxymethylcellulose barium sulfate layer.  
d Same subcoated tablet as used for C-I, C-II, and C-III series [see Table I of reference (1)].  
e A subcoated tablet with average weight of 0.8 Gm. per tablet.  
f A subcoated ammonium chloride tablet with average weight of 0.766 Gm. per tablet.

through the screen of the U. S. P. disintegration apparatus. In the case of tablets coated with ammoniated shellac, the end point was not nearly as sharp since frequently the coatings formed a sack through which the core tablet contents were leached by the fluids.

Two studies were carried out. The first study consisted of two parts: (a) a study of the dependence of acid resistance and disintegration time of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc on the pH of the test fluid, and (b) a study of the dependence of the disintegration time of tablets coated with ammoniated shellac on the pH of the test fluid. The second study was concerned with the stability of enteric coatings with respect to their disintegration behavior. Tablets with different enteric coatings were tested initially and after storage for periods of time from one to twenty-four months at 4°, room temperature, and at two elevated temperatures, 40 and 47°. In many of these tests the disintegration time was determined independently by two different laboratories, designated "A" and "B" in Tables VI and VII. In the range from fifteen to fifty minutes, in which most of the average disintegration times of the enteric coated tablets fall, the agreement between the two laboratories was excellent.

## RESULTS

**Potentiometric Titrations.**—The styrene-maleic acid copolymer was found to have an apparent  $pK_1'$  of 4.24. This was an average of five determinations which ranged from 4.20 to 4.27. Garrett and Guile (16) reported a  $pK_1'$  of 4.25 for the copolymer. The hydrolyzed Resin SC-2 was found to have an apparent  $pK_1'$  of 4.83. This was an average of four determinations which ranged from 4.70 to 4.94.

Accurate  $pK_1'$  values for starch acetate phthalate, cellulose acetate phthalate, and shellac could not be obtained in water or aqueous-organic solvent mixtures due to the tendency of the polymers to precipitate during the titrations.

**Dependence of Acid Resistance and Disintegration Time on the pH of the Test Fluid.**—Results obtained with three lots of tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc are shown in Table III.

Within experimental error, two of the lots, C 11-4 and C 11-6, exhibited the same resistance to both artificial gastric fluids, pH 1.2 and pH 3.0.

In artificial intestinal fluids, having pH values of 3.7, 4.0, and 4.8, the disintegration time of lot C 11-5 decreased as the pH increased. However, the disintegration time reached an apparent minimum at pH 4.8 because the disintegration times at pH 6.9 and 4.8 were not significantly different.

Starch acetate phthalate coatings are much less sensitive to low pH fluids than the styrene-maleic acid copolymer coating. For example, in the artificial intestinal fluid, pH 4.8, lot C 111-2 exhibited only broken coatings after two hundred and thirty minutes, whereas, as formerly reported (1) these tablets were completely disintegrated after an average time of forty-one minutes in artificial intestinal fluid, pH 6.9.

Tablets coated with ammoniated shellac require media of even higher pH in order to obtain reasonably low disintegration times. Table IV is a tabulation of disintegration times of fourteen stability samples of six different lots of tablets coated with ammoniated shellac.

The results indicate that the shellac-coated tablets disintegrate much more rapidly in simulated intestinal fluid, pH 7.5, than in artificial intestinal fluid, pH 6.9.

**Stability of Enteric Coatings With Respect to Their Disintegration Behavior.**—Results shown in Tables IV and V indicate that tablets enteric coated with shellac exhibit progressively increasing average disintegration times with increasing storage time at room temperature and at elevated temperatures.

In contrast to styrene-maleic acid copolymer alone, shellac in combination with the copolymer (lots J and K) causes a marked increase in disintegration time after storage of the tablets for one month at 47°.

The disintegration times of tablets coated with starch acetate phthalate also increase markedly with increase in storage time at room temperature and at elevated temperatures. The disintegration times increase more rapidly with age when Span 80 and propylene glycol are used with starch acetate phthalate than when dibutyl phthalate is used as a plasticizer. This difference can be seen by comparing lots L and M in Table V.

Tablets coated with a mixture of cellulose acetate phthalate and styrene-maleic acid copolymer (lot Q, Tables VI and VII) became more sensitive to simulated gastric juice after aging at room temperature and elevated temperatures.

Tablets coated with styrene-maleic acid co-

TABLE III—INITIAL DISINTEGRATION TESTS ON TABLETS COATED WITH STYRENE-MALEIC ACID COPOLYMER, DIBUTYL PHTHALATE, AND TALC IN VARIOUS SYNTHETIC GASTRIC AND INTESTINAL FLUIDS

Test Fluid	CII 4		Tablet Lot CII-5		CII 6	
	Av	S E <sup>a</sup>	Av	S E	Av	S E
Simulated gastric fluid, U. S. P., pH 1.2	165 <sup>b</sup>	8.2	193 <sup>b</sup>	8.6	273 <sup>b</sup>	12.1
Artificial gastric fluid, pH 3.0	180 <sup>b</sup>	6.6			268 <sup>b</sup>	19.7
Artificial intestinal fluid, pH 3.7			49.5 <sup>d</sup>	2.5	..	..
Artificial intestinal fluid, pH 4.0			41.6 <sup>d</sup>	1.5		
Artificial intestinal fluid, pH 4.8			24.3 <sup>d</sup>	0.67		
Artificial intestinal fluid, pH 6.9			23.8 <sup>d</sup>	0.23		

<sup>a</sup> Standard error

<sup>b</sup> Average time (in minutes) to coating attacked end point as defined previously (15)

<sup>c</sup> There are no significant differences in the pairs of averages which are bracketed.

<sup>d</sup> Average time (in minutes) for complete disintegration of the coated tablet



TABLE IV.—AVERAGE DISINTEGRATION TIMES<sup>a</sup> AND THEIR STANDARD ERRORS OF TABLETS ENTERIC COATED WITH AMMONIATED SHELLAC<sup>b</sup>

Lot	Storage Conditions of Tablets Before Test		Artificial Intestinal Fluid, pH 6.9		Simulated Intestinal Fluid, pH 7.5	
	Temperature	Time, Months	Av.	S. E.	Av.	S. E.
D	RT <sup>c</sup>	6	83	4.2	37	1.8
D	40°	3	136	4.4	78	3.4
E	RT	3	33.7	1.6	32.5	1.2
E	40°	3	103	3.8	58	2.5
E	47°	3	>155	..	>155	..
F	RT	3	50.0	3.3	22.2	0.87
F	40°	3	92	3.6	73	6.4
F	47°	3	>240	..	163	12.5
G	40°	3	>240	..	64	3.9
G	47°	3	>240	..	>240	..
H	RT	3	52.5	1.7	32.2	0.87
H	40°	3	132	7.0	50.	2.7
I	RT	3	50.0	1.7	29.8	0.82
I	40°	3	145	4.6	52.5	2.5

<sup>a</sup> Based on 6 tablets in each test fluid<sup>b</sup> Prior to the disintegration tests the tablets were unaffected by immersion in simulated gastric fluid U. S. P. for one hour.<sup>c</sup> Room temperature.TABLE V.—AVERAGE DISINTEGRATION TIMES<sup>a</sup> AND THEIR STANDARD ERRORS OF VARIOUS ENTERIC COATED TABLETS IN ARTIFICIAL INTESTINAL FLUID, pH 6.9

Lot	Enteric Substance	Initial		3 Months at RT		6 Months at RT		1 Month at 47°		3 Months at 47°	
		Av.	S. E.	Av.	S. E.	Av.	S. E.	Av.	S. E.	Av.	S. E.
J	Styrene-maleic acid copolymer plus shellac	49.2	0.9	...	..	...	...	137.7	3.9	...	...
K		33.8	0.8	...	...	...	...	149.8	8.5	...	...
L	Starch acetate phthalate	58.2	1.5	65.8	1.7	88.9	4.9	177.5	2.1	...	...
M		35.7	1.0	40.3	0.9	40.8	1.3	90	2.5	115	2.8
A	Ammoniated shellac	25.9	0.5	42.6	1.0	82.8	4.2	151	19	>240	...
B		22.5	0.8	111	6.6	...	...	170	21	>240	...

<sup>a</sup> Based on 12 tablets.TABLE VI.—AVERAGE DISINTEGRATION TIMES AND THEIR STANDARD ERRORS OF VARIOUS ENTERIC COATED TABLETS IN ARTIFICIAL INTESTINAL FLUID, pH 6.9<sup>a</sup>

Type of Coating	Lot		Initial		Storage Time at Room Temperature, months							
			Av.	S. E.	3	S. E.	6	S. E.	12	S. E.	18	24
Styrene-maleic acid copolymer	BI-1	A <sup>b</sup>	15.1	0.31	16.5	0.55						
		B <sup>c</sup>	15		15		16.8	0.27	13.2	0.14	21.8	0.44
	BII-1	A	41.0	0.80	42.5	0.74						
		B	40		40		45.0	0.43	46.0	0.82	49.8	0.93
	BII-2	A	41.3	0.71	43.4	1.1						
		B	40		40		44.2	0.49	41.7	0.58	50.7	0.71
	N	A	46.4	1.6								
		B	45		51.5	0.68	52.9	0.42	51.8	1.3	53.0	1.3
	BI-2	A	46.8	0.84	51.7	1.4						
		B	50		40		52.4	0.91	56.7	0.48	59.8	1.0
Hydrolyzed Resin SC-2	O	A	15.8	0.45	18.6	1.1						
		B	20		20		20.9	0.50	17.3	1.6	23.4	1.9
	P	A	23.8	0.80								
		B	20		24.5	0.53	24.2	0.09	29.0	0.64	29.8	0.60
Mixture of styrene-maleic acid copolymer and cellulose acetate phthalate	Q <sup>d</sup>	A	17.3	0.55	20.5	0.68						
		B	20				15.2	0.67	19.7	0.75	20.2	0.59
Double enteric coated tablets (see Table II)	R	A	52.3	0.93	53.0	1.2						
		B	45		40		41.3	0.61	41.7	0.89	44.0	1.0
	S	A	56.9	1.6	64.3	1.2						
		B	65		55		65.4	1.3	60.3	1.5	61.4	1.5
Film-coated over enteric coating (see Table II)	T	A										
		B	44.0	0.95	47.5	1.0	57.8	1.0	56.8	1.4	61.5	1.1
	U	A										
		B	61.8	0.60	53.9	0.63	62.3	0.28	74.2	1.7	69.3	1.7

<sup>a</sup> Unless otherwise indicated, all tablets tested were unaffected by immersion in simulated gastric fluid for two hours prior to the disintegration test, time in minutes.<sup>b</sup> Disintegration time determined by laboratory "A" based on 12 tablets.<sup>c</sup> Disintegration time determined by laboratory "B" based on 12 tablets.<sup>d</sup> Some of the tablets were noticeably affected by the two-hour treatment with simulated gastric fluid U. S. P.

TABLE VII.—AVERAGE DISINTEGRATION TIMES AND THEIR STANDARD ERRORS OF VARIOUS ENTERIC COATED TABLETS IN ARTIFICIAL INTESTINAL FLUID, pH 6.9<sup>a</sup>

Type of Coating	Tablet Lot	Storage Conditions											
		21 mo	at 4°	1 mo	at 47°	3 mo	at 40°	3 mo	at 47°	6 mo	at 40°		
		Av	S E	Av	S E	Av	S E	Av	S E	Av	S E		
Styrene maleic acid copolymer	BI 1	16 3 <sup>b</sup>	0 31	16 4 <sup>b</sup>	0 41	18 4 <sup>b</sup>	0 31	19 5 <sup>b</sup>	0 37	33 5 <sup>b</sup>	0 44		
	BII-1	16 3 <sup>b</sup>	0 77	53 3 <sup>b</sup>	0 60	57 6 <sup>c</sup>	1 4	61 1 <sup>b</sup>	0 77	45 8 <sup>c</sup>	0 62		
	BII 2	13 5 <sup>b</sup>	0 70	53 3 <sup>b</sup>	0 74	47 4 <sup>c</sup>	1 9	55 6 <sup>b</sup>	0 68	41 3 <sup>c</sup>	0 60		
	N	16 6 <sup>b</sup>	0 66	50 7 <sup>b</sup>	2 2	48 4 <sup>c</sup>	1 5	47 8 <sup>c</sup>	0 62	43 7 <sup>c</sup>	0 88		
	BI 2	55 0 <sup>c</sup>	0 84	65 3 <sup>b</sup>	0 73	54 4 <sup>c</sup>	0 88	73 0 <sup>b</sup>	0 64	48 5 <sup>c</sup>	0 34		
Hydrolyzed Resin SC-2	O	17 9 <sup>c</sup>	0 52	26 1 <sup>b</sup>	1 1	36 1 <sup>b</sup>	1 5	43 0 <sup>b</sup>	0 86	27 5 <sup>c</sup>	0 74		
	P	27 2 <sup>c</sup>	0 52	32 2 <sup>b</sup>	0 89	32 6	1 2	36 8 <sup>c</sup>	0 71	38 4 <sup>c</sup>	1 5		
Mixture of styrene maleic acid copolymer and cellulose acetate phthalate	Q	22 0 <sup>c</sup>	0 42	20 3 <sup>b</sup>	0 70	22 8 <sup>c,d</sup>	0 64	22 0 <sup>b</sup>	1 1	24 0 <sup>c,e</sup>	1 3		
Double enteric coated tablets (see Table II)	R	52 2 <sup>c</sup>	1 8	47 2 <sup>b</sup>	1 0	58 5 <sup>c</sup>	1 2	48 7 <sup>b</sup>	1 0	35 6 <sup>c</sup>	1 2		
	S	63 3 <sup>c</sup>	1 4	81 1 <sup>b</sup>	1 8	104 9 <sup>c</sup>	3 9	110 3 <sup>b</sup>	1 9	117 3 <sup>c</sup>	2 4		
Film coated over enteric coating (see Table II)	T	54 0 <sup>c</sup>	0 87	77 2 <sup>c</sup>	0 82	60 1 <sup>c</sup>	1 1	121 2 <sup>c</sup>	8 0	127 0 <sup>c</sup>	3 5		
	U	62 1 <sup>c</sup>	0 66	71 0 <sup>c</sup>	1 1	63 3 <sup>c</sup>	3 5	82 3 <sup>c</sup>	3 1	81.3 <sup>c</sup>	8.1		

<sup>a</sup> Unless otherwise indicated, all tablets tested were unaffected by immersion in simulated gastric fluid for two hours prior to the disintegration test, time in minutes

<sup>b,c</sup> Disintegration times determined by laboratories 'A' and 'B' respectively Twelve tablets were used in each case

<sup>d,e</sup> Two of twelve and nine of twelve tablets, respectively had broken coatings after exposure to simulated gastric fluid for two hours

polymer or with hydrolyzed Resin SC-2 exhibited only very slight increases in disintegration times after storage for prolonged periods at room temperature or at elevated temperatures. Five lots of tablets coated with styrene-maleic acid copolymer and two lots coated with hydrolyzed Resin SC-2 were tested. After storage for either two years at room temperature or one month at 47° these seven lots exhibited an average increase in disintegration time of only ten minutes in artificial intestinal fluid, pH 6.9.

The application of a film coating of sodium carboxymethylcellulose and talc over a styrene-maleic acid coating caused the disintegration times to increase upon aging of the tablets more than could be accounted for by the small increase in disintegration time due to aging of the styrene-maleic acid copolymer coating alone. This can be seen by comparing the data for lot T with that of lot BII-1 and for lot U with lot B I-2 in Tables VI and VII. The reason for this effect of the film coating is being investigated further.

None of the tablets coated with styrene-maleic acid copolymer or hydrolyzed Resin SC-2 showed any decreased acid resistance after prolonged storage at room temperature or at elevated temperatures as evidenced by a two-hour immersion in simulated gastric fluid with the standard U S P apparatus. In fact, the resistance at pH 1.2 of such coated tablets usually increases with aging, particularly at elevated temperatures. For example, lot BI-1 had an initial average resistance time of four and three-quarter hours in simulated gastric fluid. After storage for twelve months at 40° followed by four months at room temperature, the tablets were unaffected by immersion in simulated gastric fluid for seven and one-half hours at 37°, after an additional thirty-seven hours in the fluid at room temperature, the tablet coatings had only small cracks and most of the core tablets were still intact.

## DISCUSSION

Relationship Between Disintegration Rate, Apparent  $pK'$  of Enteric Substance, and pH of Test Fluid. —Both of the polyelectrolytes, styrene-maleic acid

copolymer and hydrolyzed Resin SC-2, have  $\alpha,\beta$ -dicarboxylic acid units. One of the carboxyl groups of each unit is considerably more acidic than the other and is completely ionized before the other starts to dissociate. Since we are only concerned with pH values below the neutral point (pH 7), the ensuing discussion pertains only to the more acidic carboxyl of each  $\alpha,\beta$ -dicarboxylic acid unit.

Substituting the  $pK_1'$  of 4.24 for styrene-maleic acid copolymer into the equation of Albert (17), one can calculate that approximately 99% of the carboxyl groups will be ionized at pH 6.24 but only approximately 1% will be ionized at pH 2.24. The data of Ferry, *et al.* (13), indicate that between 1 and 10% of the carboxyl groups have to be ionized before solution of the polymer occurs in the presence of dilute hydrochloric acid. When the copolymer is used as a coating agent with a plasticizer and a dusting powder, the exact pH at which the resultant coatings dissolve, or allow rapid release of the enclosed medicament, depends to some extent on the exact composition of the coating and the nature and solubility of the medicament in the core. Acid resistance of such coatings is usually good up to about pH 3 or slightly above. At about pH 3.4 and above the rate of disintegration of the coatings become quite rapid.

It would be interesting to compare the change in rate of disintegration with change in pH for different coatings. Unfortunately, we cannot readily measure the rate of disintegration of enteric coated tablets. Only the disintegration time is measured. A reasonable assumption, however, is that the disintegration rate constant,  $R$ , is inversely proportional to the average disintegration time,  $T$ , i.e.,  $R \propto 1/T$ . Hence, if we determine values of  $\Delta\left(\frac{1}{T}\right)/\Delta pH$ , we should obtain values which are directly proportional to values of  $\Delta R/\Delta pH$ . Using the data for lot C II-5 in Table III, a plot of  $1/T$ , versus pH, in the pH range 3.7 to 4.8, yields a straight line with slope,  $\Delta(1/T)/\Delta pH$ , of 0.020. At pH 3.7, 4.0, and 4.8 the more acidic carboxyls of styrene-maleic acid copolymer are 22, 36, and 78% ionized, respectively. Hence the pH range in which the disintegration rate is increasing (or disintegration time decreasing) markedly with an increase in pH,

corresponds to the pH range where the per cent ionization of the polymer is changing most rapidly, i. e., in a narrow pH range on either side of the  $pK'_1$  of the copolymer.

Nine of the lots of tablets, which were coated with ammoniated shellac, have finite and distinctly different disintegration times at pH 6.9 and 7.5 (see Table IV). The average value of  $\Delta(1/T)/\Delta pH$  calculated for these lots was 0.020. It seems reasonable to conclude that the apparent  $pK'$  of shellac lies in the range 6.9 to 7.5.

**Relationship Between Critical *In Vitro* pH Range and Enteric Properties.**—We may define the critical *in vitro* pH range of an enteric coating as the pH range in which the disintegration rate increases rapidly or the disintegration time decreases rapidly. The critical *in vitro* pH range for the styrene-maleic acid copolymer-dibutyl phthalate-talc coating on lot C II-5 was shown to be pH 3.7 to 4.8 and that for the ammoniated shellac-talc coatings to be about pH 6.9 to 7.5. The critical *in vitro* pH ranges for starch acetate phthalate and cellulose acetate phthalate coatings apparently lies between these two pH ranges, i. e., between 3.7–4.8 and 6.9–7.5.

Disintegration at such low pH values, as observed for the styrene-maleic acid coating, is new in the enteric field. However, we have shown previously (5, 15) that such coatings function as enteric coatings in the dog and human subjects. The results should, however, not be too surprising. Maltby (18) found that after administration of a protein meal to a group of 147 patients without history of gastric disease or pernicious anemia, 65% of the cases had gastric contents with pH values ranging from 1.5 to 2.5 and 86% had gastric contents with pH values ranging from 1.5 to 3.5. A great deal of evidence indicates that the intestinal contents range from pH 3.6 to pH 7.9. To ensure that the enteric coatings disintegrate soon after reaching the small intestine we believe that enteric coated tablets should disintegrate on the acid side at as low a pH as 3.7.

**The Stability of the Enteric Coatings Studied.**—Such very small changes in average disintegration time in artificial intestinal fluid, as observed for the tablets coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc, have not been previously reported for any enteric coating. The few stability tests reported for other coatings have usually involved much shorter storage times and have not included elevated temperature studies (6–12). Some enteric coatings show decreased resistance to acid solutions or the coatings crack or craze after aging (11, 12). The increase in acid resistance of styrene-maleic acid copolymer coatings upon aging is a desirable property.

Other investigators (19, 20) made similar observations as ours with respect to shellac-coated tablets. We would like to point out, however, that such increases in disintegration time do not necessarily occur when shellac is used in small quantities as a water-proofing agent before application of sugar or other coatings to tablets.

**Correlation of Average Disintegration Times of Tablets After Storage for Twenty-four Months at Room Temperature With Average Disintegration Times of the Same Tablets After Storage for One Month at 47°.**—Use of the Arrhenius equation relating the rate of reaction with temperature was

not practically possible with the enteric coated tablets tested because of the following reasons: (a) changes in disintegration time with increase in age were very small for these newer coatings, and (b) higher temperatures than 47° could not be used because reactions occurred in the coatings which were known not to occur at 47° or below. It was desirable, however, to establish a short term, high temperature stability test which would give a satisfactory estimate of the average disintegration time which would be observed if the enteric coated tablets were stored for two years at room temperature.

Based on the twelve lots of enteric coated tablets studied, it has been found that the average disintegration time observed after storage of tablets for one month at 47° gives an excellent prediction of the average disintegration time of the tablets after storage for twenty-four months at room temperature. Figure 1 shows such a relationship using the values of average disintegration time reported in Tables VI and VII. However, the correlation is improved if those average disintegration times observed by laboratory "A" are corrected to those which would be expected to be reported by laboratory "B". The first ten average disintegration times,  $T_a$ , of tablets stored for one month at 47°, as reported by laboratory "A", were transformed into the predicted disintegration times,  $\hat{T}_b$ , which would be expected to be reported by laboratory "B", using the equation  $\hat{T}_b = 0.852 T_a + 4.2$  which was based on 49 lots of tablets. The average disintegration times of the tablets after storage for twenty-four months at room temperature were then plotted against the average disintegration times of the tablets after storage for one month at 47°,  $T_b$  or  $\hat{T}_b$ .

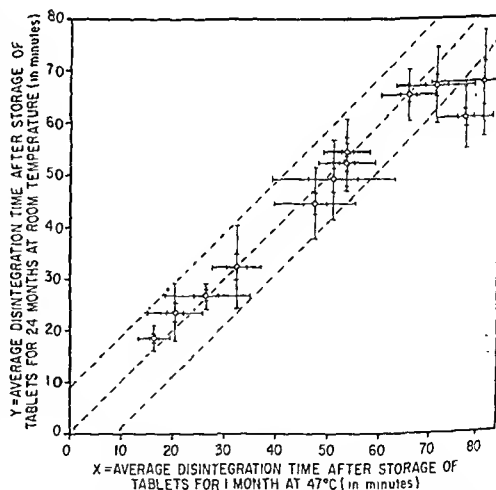


Fig. 1.—Correlation of disintegration times of twelve lots of enteric coated tablets in artificial intestinal fluid, pH 6.9, after storage for twenty-four months at room temperature with disintegration times after storage of the tablets for one month at 47°. Center dotted line represents the relation  $y = x$ . Outer dotted lines represent 95% confidence limits of the center dotted line. Inner bars on solid lines represent 95% confidence limits about the average disintegration time plotted as circles. Outer bars on solid lines represent 95% confidence limits of individual tablet disintegration times.

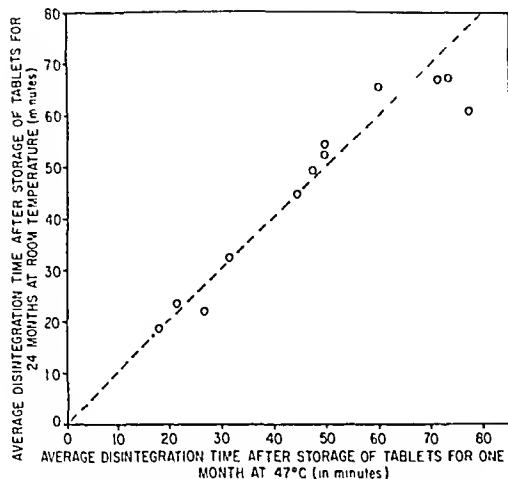


Fig 2—Correlation of disintegration times of twelve lots of enteric coated tablets in artificial intestinal fluid, pH 6.9, after storage for twenty-four months at room temperature with disintegration times after storage for one month at 47°. Average disintegration times reported by laboratory A have been converted to those which would be expected to be reported by laboratory B

The latter plot is shown in Fig 2. The "least squares" lines were shown to have a slope not differing significantly from unity. Hence, within the error of the measurements and the dispersion of the averages, the disintegration times of tablets after storage for one month at 47° is an excellent estimate of the disintegration time observed after storage for twenty-four months at room temperature.

## SUMMARY AND CONCLUSIONS

1 The resistance of tablets enteric coated with styrene-maleic acid copolymer, dibutyl phthalate, and talc to artificial gastric fluid is not significantly different at pH 1.2 or pH 3.0. In artificial intestinal fluids the disintegration time decreases over the pH range 3.7 to 4.8 but the disintegration time at pH 4.8 is not significantly different from the disintegration time at pH 6.9.

2 The average disintegration time of tablets enteric coated with either styrene-maleic acid copolymer or hydrolyzed Resin SC-2 increases only by an average of ten minutes (range four to fifteen minutes) after storage of the tablets for two years at room temperature or for one month at 47°.

3. Tablets coated with starch acetate phthalate, ammoniated shellac, or shellac plus styrene-maleic acid copolymer exhibited markedly increased disintegration times in artificial intestinal fluid, pH 6.9, after aging at room temperature or elevated temperature.

4. None of the lots of tablets coated with styrene-maleic acid copolymer or hydrolyzed Resin SC-2 showed any decreased resistance to acid at pH 1.2 after prolonged storage at room temperature or elevated temperatures as evidenced by immersion in simulated gastric fluid for two hours. After storage at elevated temperatures for prolonged periods such coated tablets exhibit markedly increased acid resistance at pH 1.2 compared with their initial acid resistance at pH 1.2.

5. Tablets coated with a mixture of cellulose acetate phthalate and styrene-maleic acid copolymer exhibited decreased acid resistance on storage at room temperature and at elevated temperatures.

6. The average disintegration time observed after storage of enteric coated tablets for one month at 47° was shown to give an excellent prediction of the average disintegration time of the tablets after storage for two years at room temperature. The correlation was based on twelve lots of tablets and included five different types of coatings.

## REFERENCES

- (1) Wagner, J. G., and Long, S., *THIS JOURNAL*, 49, 121(1960).
- (2) Wruble, M., *ibid*, 24, 570(1935).
- (3) Malm, C. J., Emerson, J., and Hiatt, G. D., *ibid*, 40, 520(1951).
- (4) Wagner, J. G., Brignall, T. W., and Long, S., *ibid*, 48, 244(1959).
- (5) Wagner, J. G., Veldkamp, W., and Long, S., *ibid*, 49, 128(1960).
- (6) Seoville, W. L., *ibid*, 4, 1241(1915).
- (7) Bukey, F. S., and Rhodes, P., *ibid*, 22, 1253(1933).
- (8) Cooper, W., *Pharm J*, 150, 101(1943).
- (9) Sampson, J. J., Foreman, H., and Solomon, B. C., *Circulation*, 5, 534(1952).
- (10) Bauer, C. W., and Geraughty, R. J., *THIS JOURNAL*, *Pract Pharm Ed*, 14, 504(1953).
- (11) Stoklosa, M. J., and Ohmart, L. M., *ibid*, 14, 507(1953).
- (12) Abbott Laboratories, British pat. specification 760 403, October 31, 1950.
- (13) Ferry, J. D., Udy, D. C., Wu, F. C., Heckler, G. E., and Fordyce, D. B., *J. Colloid Sci*, 6, 429(1951).
- (14) Wruble, M., U. S. patent 1,907,203, May 2, 1933.
- (15) Wagner, J. G., Veldkamp, W., and Long, S., *THIS JOURNAL*, 47, 681(1958).
- (16) Garrett, E. R., and Guile, R. L., *J. Am. Chem. Soc.*, 73, 4533(1951).
- (17) Albert, A., *Pharmacol. Revs.*, 4, 136(1952).
- (18) Maltby, E. J., *J. Clin. Invest.*, 13, 193(1934).
- (19) Tarnowski, C. E., *Am. Rev. Tuberc. Pulmonary Diseases*, 76, 159(1957).
- (20) Kuever, R. A., and Maney, P. V., U. S. pat. 2,373,763, April 17, 1945.

# The Preparation and Antibacterial Action of Metal Chelates of Some Antitubercular Agents, Amino Acids, and Peptides\*

By FREDERICK T. COUNTER, Jr., RONALD N. DUVALL, WILLIAM O. FOYE, and  
RAYMOND W. VANDERWYK

A series of eighteen metal chelates and complexes of some  $\alpha$ -amino acids, peptides, isonicotinoyl hydrazide, *p*-aminosalicylic acid, *p*-acetylaminobenzal thiosemicarbazone, and biotin were screened for antibacterial activity against ten microorganisms. Quantitative tests were carried out on six of these compounds by a modified serial tube dilution method; these compounds were the copper complex and chelate of PASA, cobalt chelates of INH and methionine, and copper chelates of glycyl-DL-alanine and biotin. Evidence indicated that the complexes of methionine and biotin were more active than the metal ions, but the action of the chelates of the antitubercular agents appeared to depend in part on dissociation of the chelates.

THE ACTION of chelating agents against bacteria has received some attention, but the question as to the dependence of this activity on the chelating agent or the chelate itself has not been thoroughly resolved. Erlenmeyer and workers (1), for instance, found a sizable number of compounds capable of metal chelation to have antibacterial and antituberculous activity in the presence of definite concentrations of certain metal ions. Albert (2) has investigated the antibacterial action of 8-hydroxyquinoline and provided good evidence that this agent exerts its effect only in the form of toxic metal chelates. Cymerman-Craig and Rubbo (3) have shown that isonicotinoyl hydrazide (INH) derivatives incapable of metal chelation have no antituberculous action, and Foye and Duvall have shown both *p*-aminosalicylic acid (PASA) (4) and INH (5) to be as effective against tubercle bacilli in the form of certain metal chelates. Garattini and Leonardi (6) have also shown that among a large series of compounds having inhibitory effects against *M. tuberculosis*, the most effective were two powerful chelating agents, *o*-phenanthroline and sodium diethyldithiocarbamate, which could hardly be expected to exist in aqueous media without chelating any metal ions present.

It was therefore felt that antibacterial testing of a number of metal chelates of agents having little or no antibacterial activity might be illuminating. Although positive results from this experiment would not necessarily explain the action of individual antibacterials, they would indicate that the metal chelate structure, *per se*, is capable of inhibiting the growth of

microorganisms. Among the metal chelates selected for this purpose, several antitubercular agents, which show little activity against other bacteria, were included in the hope that further information regarding the importance of chelation in their action against tubercle bacilli might be found.

## METHODS

**Materials.**—The PASA used was a gift of Parke, Davis and Co., the INH was a gift of the Massachusetts General Hospital, and the cupric, ferric, and zinc oxides were Baker's C. P. analyzed reagents. The preparation of the copper complex and copper and iron chelates of PASA (4), and the copper, iron, zinc, and cobalt chelates of INH (5) has already been described. The preparations of the remainder of the chelates follow.

***p*-Aminobenzaldehyde Thiosemicarbazone.**—This compound was synthesized by the method of Beard and Hodgson (7). A 65% yield of product was obtained which melted at 196–197° [lit. m. p. 198° (8)].

***p*-Acetylaminobenzaldehyde Thiosemicarbazone (Tibione).**—The acetylation procedure of Behnisch, *et al.* (9), was used. A 52% yield of product was obtained which melted at 228–229° [lit. m. p. 230° (9)].

**Copper Chelate of *p*-Acetylaminobenzaldehyde Thiosemicarbazone.**—This was prepared by the slow addition, with stirring, of 1.88 Gm. (0.0075 mole) of copper sulfate pentahydrate in 400 ml. of water to 3.54 Gm. (0.015 mole) of *p*-acetylaminobenzaldehyde thiosemicarbazone in 500 ml. of hot absolute ethanol. A yellow-green compound precipitated immediately as the pH dropped from 5.2 to 2.6. The mixture was stirred until it had cooled to room temperature and was filtered by gravity. The precipitate was washed free of sulfate ion and was air dried. The yield was 3.45 Gm. (80.8% based on a 2:1 chelate ratio).

**Anal.**—Calcd. for  $C_{20}H_{22}CuN_8O_5S_2 \cdot 2H_2O$ : Cu, 11.15;  $H_2O$ , 6.32. Found: Cu, 11.83, 11.74 (by ashing);  $H_2O$ , 6.42 (by wt. loss on drying).

Sulfate ion was found to be absent by barium ion test. The saturated aqueous solution gave a nega-

\* Received August 21, 1959, from the Departments of Chemistry and Biological Sciences, Massachusetts College of Pharmacy, Boston.

Abstracted in part from a thesis submitted by F. T. Counter, Jr., as a requirement for the degree of Master of Science, 1958.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

tive test for cupric ion with ferrocyanide ion. The test for cupric ion was still negative after the chelate was suspended for one hour in dilute hydrochloric acid (pH 3.0).

**Cobalt Chelate of Methionine**—An aqueous solution of 1.19 Gm. (0.005 mole) of cobaltous chloride hexahydrate was added slowly, with stirring, to an aqueous alkaline solution of 1.5 Gm. (0.01 mole) of DL-methionine. The pH dropped from 7.9 to 4.7. The resulting solution was made alkaline, and a pink-brown precipitate was isolated at a pH of 10.4, and was washed free of chloride ion and air dried. The yield was 1.7 Gm. (96% based on a 2:1 chelate structure). Water of hydration was absent.

*Anal*—Calcd for  $C_{10}H_{20}CoN_2O_4S_2$ : Co, 16.60. Found: Co, 17.71.

**Copper Chelate of Methionine Sulfoxide**—To an aqueous solution of 0.825 Gm. (0.005 mole) of methionine sulfoxide [prepared by the method of Toennies and Kolb (10)] was added 0.488 Gm. (0.005 mole) of cupric hydroxide. The mixture was stirred for three hours, the excess cupric hydroxide was removed by filtration, and the filtrate was concentrated on a steam bath to near dryness. Absolute ethanol was added, and the resulting purple compound was isolated and washed with absolute ethanol. The yield, after air drying, was 0.97 Gm. (99% based on a 2:1 chelate structure). Water of hydration was absent.

*Anal*—Calcd for  $C_{10}H_{20}CuN_2O_6S_2$ : Cu, 16.22. Found: Cu, 14.78.

**Cobalt Chelate of Cystine**—Sufficient dilute sodium hydroxide solution was added to just dissolve 2.4 Gm. (0.01 mole) of L-cystine. To this solution was added slowly with stirring 2.38 Gm. (0.01 mole) of cobaltous chloride hexahydrate in a minimum amount of water. A pink precipitate formed immediately, and the mixture jelled when addition was complete. The pH dropped from 9.8 to 6.7. The mixture was stirred for an hour, filtered, washed free of chloride ion, and air dried. The yield was 3.1 Gm. (98% based on a 1:1 chelate structure).

*Anal*—Calcd for  $C_6H_{10}CoN_2O_6S_2$ : Co, 18.70,  $H_2O$ , 5.72. Found: Co, 17.20,  $H_2O$ , 4.03.

**Ferrous Chelate of Cystine**—A solution of 2.4 Gm. (0.01 mole) of L-cystine was made in dilute sodium hydroxide solution to give a pH of 9.3. An aqueous solution of 2.78 Gm. (0.01 mole) of ferrous sulfate heptahydrate was added slowly, with stirring. A yellow precipitate formed as the pH dropped from 9.3 to 6.2 and changed to an orange-brown as the pH dropped to 5.6. The precipitate was filtered, washed free of sulfate ion with water, and air dried. The yield was 2.73 Gm. (93% based on a 1:1 chelate structure). Sulfate ion was found to be absent while 1.6% moisture was found on drying.

*Anal*—Calcd for  $C_6H_{10}FeN_2O_6S_2$ : Fe, 19.01. Found: Fe, 16.87.

**Copper Complex of Biotin**—Dilute sodium hydroxide solution was added, dropwise, to an aqueous suspension of 0.88 Gm. (0.0036 mole) of D-biotin until solution resulted. An aqueous solution of 0.9 Gm. (0.0036 mole) of copper sulfate pentahydrate was added slowly, with stirring. The pH dropped from 8.2 to 5.0 with the formation of a green precipitate. The yield after filtering, washing, and air drying was 0.425 Gm. (39% based on a 3:2 chelate structure). Sulfate ion was found to be absent.

*Anal*—Calcd for  $C_{20}H_{34}CuN_4O_8S_3 \cdot 4H_2O$ : C, 38.80; H, 5.64; Cu, 13.69;  $H_2O$ , 7.76. Found: C, 38.09; H, 5.81; Cu, 12.35 (by titration), 12.01 (by ashing),  $H_2O$ , 8.92 (by drying), 8.93 (by Karl Fischer determination).

**Copper Derivative of Phthaloylglycine**—An aqueous solution of 1.25 Gm. (0.005 mole) of copper sulfate pentahydrate was added slowly, with stirring, to 2.05 Gm. (0.01 mole) of phthaloylglycine [prepared by the method of Billman (11)] dissolved in dilute sodium hydroxide solution to give a pH of 7.5. A blue precipitate formed with an accompanying pH drop to 4.8. The product was filtered, washed with water, and air dried. The yield was 1.78 Gm. (68% based on a 2:1 ratio). Sulfate ion was found to be absent.

*Anal*—Calcd for  $C_{20}H_{12}CuN_2O_8 \cdot 3H_2O$ : C, 45.67; H, 3.43; Cu, 12.09;  $H_2O$ , 10.28. Found: C, 44.63; H, 2.85; Cu, 11.44 (by titration), 11.18 (by ashing),  $H_2O$ , 10.11 (by drying), 10.60 (by Karl Fischer determination).

**Copper Chelate of Phthaloylglycylglycine**—To an aqueous suspension of 0.87 Gm. (0.0033 mole) of phthaloylglycylglycine [prepared by the procedure of Emerson (12)] was added dilute sodium hydroxide solution until solution resulted. To this solution was added slowly, with stirring, 0.42 Gm. (0.0017 mole) of copper sulfate pentahydrate in water. The pH dropped from 6.0 to 5.1 and a pale blue precipitate appeared. The yield, after filtration, washing free of sulfate ion, and air drying, was 0.65 Gm. (60% based on a 2:1 chelate structure).

*Anal*—Calcd for  $C_{24}H_{18}CuN_4O_{10} \cdot 4H_2O$ : Cu, 9.66;  $H_2O$ , 10.95. Found: Cu, 8.77;  $H_2O$ , 9.98 (by drying).

During drying a color change to green occurred. Sulfate ion was found to be absent.

**Diphthaloylglycylcystine**—Thirty-two grams of sodium bicarbonate and 9.6 Gm. (0.04 mole) of L-cystine were mixed with 300 ml. of water. To this suspension was added slowly, with stirring, 17.9 Gm. (0.08 mole) of phthaloylglycyl chloride [made by the procedure of Emerson (12)] dissolved in 400 ml. of benzene. The mixture was stirred for one hour after the addition and the aqueous phase was separated, filtered, and made acid. The precipitate was filtered and dried, and a yield of 6.35 Gm. (26%) of product melting at 152–155° was obtained. Water was found to be present in the compound.

*Anal*—Calcd for  $C_{26}H_{22}N_4O_{10}S_2 \cdot 2H_2O$ : C, 48.00; H, 4.03. Found: C, 46.53; H, 3.84.

**Copper Chelate of Diphthaloylglycylcystine**—Dilute sodium hydroxide solution was added, dropwise, to an aqueous suspension of 2.04 Gm. (0.0032 mole) of diphthaloylglycylcystine until solution had taken place. To this was added slowly, with stirring, 0.84 Gm. (0.0033 mole) of aqueous copper sulfate pentahydrate. The pH dropped from 6.6 to 4.4 with the formation of a green precipitate, which was collected, washed free of sulfate ion, and air dried. The yield was 1.25 Gm. (57% based on a 1:1 chelate structure).

*Anal*—Calcd for  $C_{26}H_{20}CuN_4O_{10}S_2 \cdot 3H_2O$ : Cu, 8.70;  $H_2O$ , 7.40. Found: Cu, 8.13 (by ashing);  $H_2O$ , 7.41 (by drying).

**Copper Chelate of Glycyl-DL-alanine**—A solution of 330 mg. (0.0023 mole) of glycyl-DL-alanine (Mann Fine Chemicals) in 50 ml. of water was stirred for

TABLE I—ANTIBACTERIAL SCREENING OF THE METAL CHELATES

Compound	Zone of Inhibition mm									
	Org → <sup>a</sup>	A	B	C	D	E	F	G	H	I
(PAS) <sub>2</sub> Cu complex		11	8	11	3	3	4	6	16	4
(PAS) <sub>2</sub> Cu chelate		8	6	7	3	3	4	3	9	2
(PAS) <sub>2</sub> Fe		0	0	2	2	0	0	0	0	0
INH Cu		4	4	7	6	2	1	4	3	0
INH Fe		3	0	8	2	2	3	4	7	2
INH Zn		3	4	8	4	2	2	7	7	3
(INH) <sub>2</sub> Co		14	11	19	9	8	8	16	13	13
(INH) <sub>2</sub> Cu		2	0	3	0	4	2	3	2	0
(Tibione) <sub>2</sub> Cu		0	0	7	0	2	0	0	2	0
Biotin Cu		8	7	12	9	7	8	6	10	7
Methionine Co		14	10	17	10	8	14	17	12	11
Cystine Fe		0	0	0	0	0	0	0	0	0
Cystine Co		0	0	0	0	0	0	0	0	0
Methionine sulfoxide Cu		5	2	4	0	3	5	3	0	0
Phthaloylglycine Cu		4	9	5	3	3	3	6	4	4
Glycylalanine Cu		8	14	12	7	6	11	12	4	8
Diphthaloylglycylcystine Cu		2	7	2	0	2	1	2	2	0
Phthaloylglycylglycine Cu		3	2	2	0	3	1	2	2	5
PASA		7	5	12	4	2	7	2	0	0
INH		2	2	2	16	3	7	2	0	0
Tibione		0	0	0	0	0	0	0	0	0
CuO		0	0	0	0	0	0	0	0	0
Fe <sub>2</sub> O <sub>3</sub>		0	0	0	0	0	0	0	0	0
ZnO		1	1	6	0	0	1	4	4	0

<sup>a</sup> Test organisms A—*E. coli* B—*S. marcescens* C—*S. albus* D—*K. pneumoniae* E—*P. vulgaris* F—*B. subtilis* G—*A. faecalis* H—*S. aureus* I—*A. aerogenes* J—*P. aeruginosa*

three hours with an excess of cupric hydroxide. The excess cupric hydroxide was removed by filtration, and the filtrate was concentrated to near dryness on a steam bath. Absolute ethanol was added, and the resulting deep blue crystals were filtered and washed with absolute ethanol. The yield was 400 mg (95% based on a 2:1 chelate structure).

Anal.—Calcd for C<sub>10</sub>H<sub>18</sub>CuN<sub>4</sub>O<sub>6</sub>·H<sub>2</sub>O: Cu, 17.07; H<sub>2</sub>O, 4.84. Found: Cu, 17.85 (by ashing), H<sub>2</sub>O, 7.18 (by drying).

**Antimicrobial Screening.**—The agar plate method of the U. S. Dept. of Agriculture (13) was employed with the following organisms: *Escherichia coli*, *Serratia marcescens*, *Staphylococcus aureus*, *Staphylococcus albus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Alcaligenes faecalis*, *Aerobacter aerogenes*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. The results of the screening are shown in Table I. Duplicate determinations were made, and the zones of inhibition were measured from the edge of the compound, which was insoluble in water in all cases, to the edge of growth.

**Quantitative Determinations.**—The six most promising compounds from the screening were tested quantitatively against *S. aureus* and *E. coli* by a serial tube dilution method. Each chelate (100 mg, except in the case of biotin copper chelate, where 22 mg was used) was added to 100 ml of sterile distilled water and placed in an incubator at 37°. The mixtures were shaken occasionally during forty-eight hours, by which time they had become homogeneous. A molecular equivalent of the parent compounds and of the corresponding metal salts, copper sulfate, and cobaltous chloride, was also dissolved in 100 ml of sterile distilled water. All solutions were filtered through a sterile Berkefeld filter, and 50 ml of each solution was serially diluted. To all solutions was added 0.1 ml of a 1:100 dilution of a twenty-four hour broth culture of test organism.

After inoculation, all solutions were incubated at 37°, and at intervals of one, two, four, and eight hours, a 4 mm loopful was transferred to a culture tube containing 5 ml of nutrient broth. These subculture tubes were then incubated for ninety-six hours at 37° and were examined visually for the presence of growth. The highest effective dilution of each chelate and control compound is shown in Table II.

## RESULTS AND DISCUSSION

As a result of the agar plate screening procedure (Table I) six of the most active chelates were selected for the determination of bacteriostatic end points by serial tube dilution in nutrient broth. The compounds so determined were the (PAS)<sub>2</sub> copper complex (4), (PAS)<sub>2</sub> copper chelate (4), (INH)<sub>2</sub> cobalt chelate (5), glycyl DL alanine copper chelate, methionine-cobalt chelate, and biotin copper chelate. There is some question whether the biotin copper compound, isolated as a 3:2 combination of biotin to copper ion, is actually a cyclic chelate or an open complex, since none of the usual criteria for chelation serve to distinguish the two in this case. The significant growth inhibitory differences between this compound and copper ion indicate that the product is not a salt.

Examination of Table II, showing the greatest dilutions at which growth inhibition of *E. coli* and *S. aureus* in nutrient broth was noted, reveals that the (PAS)<sub>2</sub>-copper complex was devoid of activity, whereas the copper chelate was active. It was less active toward *E. coli* than either PASA or copper ion, but against *S. aureus* was more active than PASA, and by the end of eight hours was approaching the activity of copper ion. The (INH)<sub>2</sub> cobalt chelate was considerably more active than INH but distinctly less active than a molar equivalent of cobalt

TABLE 11.—INHIBITORY CONCENTRATIONS OF SEVERAL METAL CHELATES *in Vitro*

Compound	Hr →	<i>E. coli</i> , M/1,000				<i>S. aureus</i> , M/1,000			
		1	2	4	8	1	2	4	8
(PAS) <sub>2</sub> Cu complex									
PASA		1 04	0 13	0 13	0 13		4 17	1 04	1 04
CuSO <sub>4</sub> ·5H <sub>2</sub> O		0 15	0 07	0 07	0 07		0 07	0 07	0 07
(PAS) <sub>2</sub> Cu chelate		2 60	2 60	2 60	0 32		1 30	0 65	0 16
PASA		0 65	0 16	0 16	0 08		5 16	2 58	1 29
CuSO <sub>4</sub> ·5H <sub>2</sub> O		0 18	0 09	0 09	0 09		0 05	0 05	0 05
(INH) <sub>2</sub> Co		1 35	0 04	0 01	0 005	0 62	0 62	0 31	0 08
INH		3 12	0 20	0 20	0 025				
CoCl <sub>2</sub> ·6H <sub>2</sub> O		0 15	0 02	0 002	0 001		0 15	0 04	0 009
Glycylalanine Cu				1 38	0 04		0 34	0 17	0 08
Glycylalanine									
CuSO <sub>4</sub> ·5H <sub>2</sub> O		0 09	0 05	0 05	0 05		0 05	0 05	0 05
Methionine Co					0 01	0 70	0 02	0 005	0 005
Methionine					2 75		2 75	0 02	0 01
CoCl <sub>2</sub> ·6H <sub>2</sub> O		0 18	0 05	0 001	0 001		0 09	0 02	0 005
Biotin Cu		0 007	0 003	0 003	0 003				0 003
Biotin									
CuSO <sub>4</sub> ·5H <sub>2</sub> O		0 09	0 05	0 05	0 05		0 04	0 04	0 04

\* A dash indicates that no inhibition of growth was observed at a concentration of 100 mg % of chelate or its molar equivalent in the case of the chelating agent and metal ion

ion against both organisms. Glycyl-DL-alanine-copper chelate reached the activity of copper ion in eight hours, although it was much less active in the shorter periods of time where observations were made. Methionine-cobalt chelate, however, presents a different picture. Against *E. coli* it showed no effect until eight hours had passed, when it had one-tenth the activity of cobalt ion, against *S. aureus* the chelate showed greater activity than cobalt ion until eight hours had passed, when the two activities became equal. The biotin-copper complex shows a fifteen-fold greater activity against both organisms than copper ion alone.

It appears in the cases of (PAS)<sub>2</sub>-copper chelate, (INH)<sub>2</sub>-cobalt chelate, and glycyl-DL-alanine-copper chelate that the antibacterial action is parallel to, or at least increases with, the partial dissociation of the chelate. The activity cannot be due wholly to dissociation, however, or the (PAS)<sub>2</sub>-copper complex, which is less stable than the chelate (4), would show greater activity than the chelate. The action of the methionine-cobalt chelate on the other hand does not seem to parallel that of the cobalt ion. Here, no activity is found against *E. coli* until eight hours have passed, which is true likewise for methionine itself, while the action against *S. aureus* increases with time but is greater than that of cobalt ion until the eight-hour observation period. It is improbable that the quantities of these compounds employed would affect the pH of nutrient broth sufficiently to cause different rates of dissociation of the chelates, and the growth of the two organisms does not provide different conditions of acidity (both cultures in the broth had a pH of 6.9).

In the case of the biotin-copper complex we have a clear-cut example of a complex showing decidedly greater activity than the metal ion. Against one organism, the activity does not materially increase with time, whereas against the other, no activity is

seen for eight hours, at which time it equals the activity shown by the other organism.

Some evidence has therefore been obtained, in the case of the biotin and methionine complexes, that a metal chelate or complex of a nonbacteriostatic agent can exert a distinctly greater growth inhibitory effect than a molecular equivalent of the metal ion. It appears, however, in the case of the antitubercular agents, PASA and INH, as well as Tibione, that the antibacterial activity of their metal chelates is dependent in part on a partial dissociation of the chelate to give metal ion. It may then be the case that formation of a metal chelate of these agents may increase the liposolubility sufficiently to aid in the passage of the cell wall (4, 5) and the chelate then dissociates within the cell, liberating toxic components. Whether free metal ion can exist within the cellular environment (without undergoing chelation) or even in the nutrient broth employed in these experiments in sufficient quantity to exert a toxic effect is a question still unanswered.

## REFERENCES

- (1) Erlenmeyer, H., Baumber, J., and Roth, W., *Helv Chim. Acta*, 36, 941(1953)
- (2) Albert, A., Gibson, M. I., and Rubbo, S. D., *Brit. J. Exptl. Pathol.*, 34, 119(1953)
- (3) Cymerman-Craig, J., Willis, D., Rubbo, S. D., and Edgar, J., *Nature*, 176, 34(1955)
- (4) Foye, W. O., and Duvall, R. N., *THIS JOURNAL*, 47, 282(1958)
- (5) Foye, W. O., and Duvall, R. N., *ibid.*, 47, 285(1958)
- (6) Garattini, S., and Leonardi, A., *Giorn. ital. chimetrap.*, 2, 18(1955)
- (7) Beard, H. G., and Hodgson, H. H., *J. Chem. Soc.*, 1944, 4
- (8) Wander, A. G., U. S. pat. 2,530,430 (1950)
- (9) Behnisch, R., Mietzsch, F., and Schmidt, H., *Am. Rev. Tuberc.*, 61, 1(1950)
- (10) Toennies, G., and Kolb, J. J., *J. Biol. Chem.*, 128, 399 (1939)
- (11) Bullman, J. H., and Harting, W. F., *J. Am. Chem. Soc.*, 70, 1473(1948)
- (12) Emerson, O. H., U. S. pat. 2,498,665 (1950)
- (13) Ruehle, G. L. A., and Brewer, C. M., U. S. Dept. Agr. Circ. No. 198(1931)



# The Effect of Various Ointment Bases on the Percutaneous Absorption of Salicylates I\*

## Effect of Type of Ointment Base

By MOISE E. STOLAR†, G. VICTOR ROSSI, and MARTIN BARR

An approach to the evaluation of the percutaneous absorption of drugs from ointment bases is described. Salicylic acid and sodium salicylate were selected as test drugs for incorporation into ointment bases representing each of four physical types of ointment vehicles. These ointments were applied to the intact shaved skin of the rabbit and confined by a specially designed bandage. Concentration of salicylate in the blood was determined at hourly intervals following application of the ointment. Salicylic acid was most effectively absorbed from hydrophilic ointment. The extent of absorption from the other ointments studied, in decreasing order, was: hydrophilic petrolatum containing water, petrolatum, and polyethylene glycol ointment. Sodium salicylate was also absorbed to the greatest extent from hydrophilic ointment, although the degree of absorption was considerably less than that observed with salicylic acid. On the basis of salicylate blood levels, no significant absorption of sodium salicylate occurred from ointments prepared with petrolatum, *hydrophilic petrolatum containing water, or polyethylene glycol ointment.*

**A**LTHOUGH a significant degree of percutaneous absorption is essential to the therapeutic usefulness of relatively few drugs applied topically in the form of ointments, it is nevertheless desirable to know the extent of such absorption from the various types of ointment bases. Such information is especially important in those cases in which absorption of drugs through the skin may lead to untoward reactions, sensitization, etc.

It was the primary aim of this study to develop a standardized procedure for determining the relative degree of absorption of drugs through the skin, and for evaluating the various factors which influence percutaneous absorption.

This report is concerned with the influence of the type of ointment base on the percutaneous absorption of salicylic acid, a predominantly oil-soluble drug, and sodium salicylate, a predominantly water-soluble drug. A subsequent report will consider the effect of various surface-active agents on the absorption of these drugs from four different physical types of ointment bases.

### EXPERIMENTAL

**Preparation of Ointments**—Salicylic acid and sodium salicylate, previously reduced to a fine powder in a ball mill and sifted through a No. 80 sieve, were incorporated in ointment bases representing each of four physical types. The bases selected were: petrolatum U. S. P. XV, an oleaginous base,

hydrophilic petrolatum U. S. P. XV in which water was incorporated, a water-in-oil emulsion base, hydrophilic ointment U. S. P. XV, an oil-in-water base, and polyethylene glycol ointment U. S. P. XV, a water-soluble base. Salicylic acid ointments were prepared to contain 6% of the active ingredient and sodium salicylate ointments were prepared to contain 6.95% sodium salicylate, which is equivalent to 6% salicylic acid.

**Analysis of Salicylic Acid Content of Ointments**—An accurately weighed sample (2 Gm.) of salicylic acid ointment was transferred to a 100-ml. beaker to which was subsequently added 25 ml. of ethyl alcohol warmed to 65° and previously neutralized with sodium hydroxide to phenolphthalein. The mixture was stirred until the ointment first melted and then congealed. The supernatant liquid was decanted and the procedure repeated. In those cases where the ointment was miscible with alcohol, the sample was dissolved in 50 ml. of neutralized alcohol. The salicylic acid content of the supernatant liquids from both extractions, or solution of the ointment base in alcohol, was determined according to the U. S. P. XV assay for salicylic acid. To compensate for possible interference from the various components in the ointment base, a blank composed of the vehicle without salicylic acid was analyzed concurrently.

**Analysis of Sodium Salicylate Content of Ointments**—A 2-Gm. sample of sodium salicylate ointment, accurately weighed, was transferred to a 100-ml. beaker. Sodium salicylate was either extracted with two 12.5-ml. portions of water warmed to 65°, or dissolved along with the base in 25 ml. of warmed water. The extraction was achieved by alternate melting and congealing of the ointment while being constantly stirred. The sodium salicylate content of the supernatant liquids from both extractions, or the aqueous solution of the ointment, was determined according to the U. S. P. XV assay for sodium salicylate. To compensate for possible interference from the ingredients of the ointment base, a blank composed of the vehicle without sodium salicylate was analyzed concurrently.

\* Received July 16, 1959, from the Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

† Abstracted from a thesis submitted by Moise E. Stolar to the faculty of the Philadelphia College of Pharmacy and Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

† Present address: Warner Lambert Research Institute, Morris Plains, N. J.

The authors wish to thank Chas. Pfizer and Co. for their financial support of this study.

All salicylic acid ointments assayed to contain 5.90-6.05% salicylic acid; all sodium salicylate ointments assayed to contain 6.80-7.10% sodium salicylate, equivalent to 5.86-6.12% salicylic acid

**Test Animal.**—Male New Zealand white rabbits weighing between 3.0 and 3.6 Kg. were used throughout the study. The animals were maintained on Purina rabbit pellets and water *ad libitum* and housed individually in temperature and humidity controlled quarters. All experiments were performed in these same quarters.

**Application of Ointment.**—Hair was removed<sup>1</sup> from the skin of the dorsal area between the forelegs and hind-legs on both sides of the spine twenty-four hours prior to application of the ointment

A bandage was devised to restrict and control the area of contact between the ointment and the skin of the rabbit. The edges of an 8.35 × 14.7 cm. piece of aluminum foil were doubled over and flattened 1 cm on each side to produce a rectangular plate measuring 6.35 × 12.7 cm. with a 1-cm. reinforced margin. An accurately weighed 7.5-Gm. sample of ointment was uniformly spread over one surface of the plate, whose opposite side was centered on a 7.5 × 20 cm strip of adhesive tape. The entire assembly was then applied to the shaved skin of the rabbit and carefully adjusted to conform to the contours of the area. To minimize contamination and insure adequate contact between the ointment and the skin, this assembly was covered with an Ace elastic bandage and fastened with strips of gauze bandage around the forelegs and hind-legs of the animal. The various components of the bandage are illustrated in Fig. 1, and the appearance of the bandage applied to the rabbit's skin is illustrated in Fig. 2. The ointment remained in contact with the skin for the nine-hour experimental period.

**Procedure.**—Due to time limitations, not more than seven animals could be utilized during any one experimental day. Therefore, on each of ten consecutive experimental days, ointments containing either salicylic acid or sodium salicylate prepared with the various type ointment bases were studied together with a salicylic acid ointment prepared from hydrophilic ointment U. S. P. XV, as a control.

One and one-half milliliters of blood were withdrawn from the marginal ear vein of the rabbit prior to, and at hourly intervals for nine hours after application of ointment. The blood samples were allowed to clot at room temperature and were then chilled in a refrigerator. After centrifugation at 2,500 r. p. m. for thirty minutes, the serum was analyzed for salicylate according to the method described by Ungar, *et al.* (1). The results obtained from a group of ten animals treated with each ointment base were analyzed by analysis of variance and the "t" test (2).

## RESULTS

The relative extent of absorption through the intact rabbit skin of salicylic acid incorporated in hydrophilic ointment, petrolatum, hydrophilic petrolatum containing water, and polyethylene glycol ointment, as determined by blood salicylate concentration (results expressed as mg. % salicylic acid), is



Fig 1.—Components of the bandage used in application of test ointments to the shaved rabbit's skin.



Fig 2—Appearance of bandage assembly in position on the rabbit.

presented in Fig 3. The results of a similar study conducted with sodium salicylate are illustrated in Fig 4. Each point on the graph represents an average of the determinations on groups of ten rabbits.

On the basis of the mean hourly blood levels of salicylate, as reported in Figs. 3 and 4, it is apparent that the type of ointment base exerts a definite influence on the extent of percutaneous absorption of both salicylic acid and sodium salicylate. It may also be noted, however, that the effect of the ointment base is considerably greater in regard to the absorption of salicylic acid as compared to sodium salicylate.

Salicylic acid was absorbed through the skin to a greater extent from hydrophilic ointment than from the other types of bases investigated. The extent of absorption of salicylic acid from the ointment bases studied, in decreasing order, was found to be: hydrophilic ointment, hydrophilic petrolatum, and petrolatum. Statistical analyses indicated a significant difference among the average blood salicylate concentrations obtained after application of each of these ointments. As measured by blood salicylate level, the degree of absorption of salicylic acid from polyethylene glycol ointment was negligible.

According to the criterion employed in this study, percutaneous absorption of salicylic acid and sodium salicylate occurred maximally between the fourth and sixth hour after application of the ointment to the skin of the experimental animal. It is, of course,

<sup>1</sup> Oster electric animal clipper, model A-2, Ang-Ra No. 2 head.

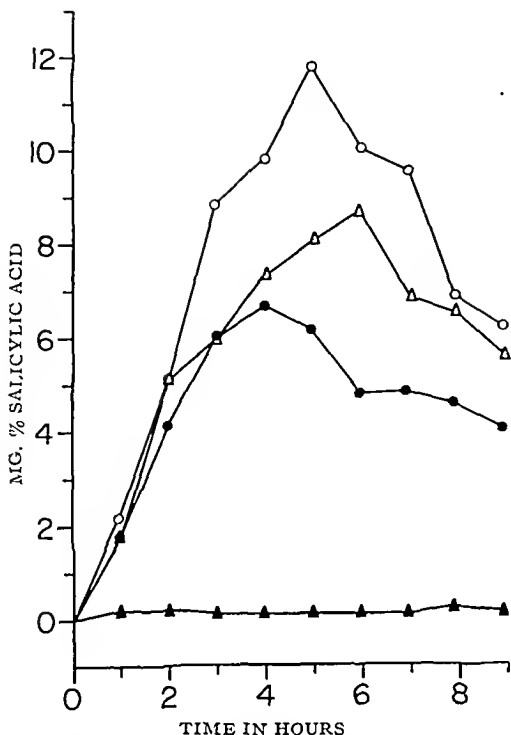


Fig. 3.—The effect of the type of ointment base on the percutaneous absorption of salicylic acid. O, hydrophilic ointment; Δ, hydrophilic petrolatum with water; ●, petrolatum; ▲, polyethylene glycol ointment.

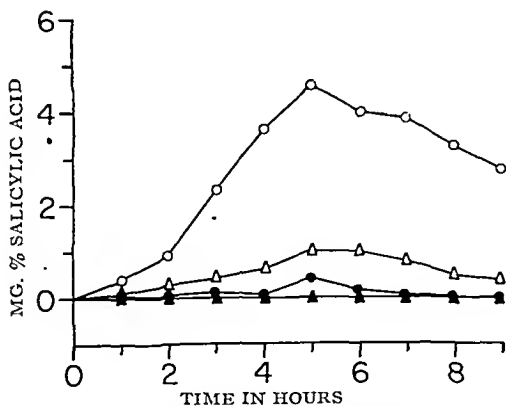


Fig. 4.—The effect of the type of ointment base on the percutaneous absorption of sodium salicylate. O, hydrophilic ointment; ●, hydrophilic petrolatum with water; Δ, petrolatum; ▲, polyethylene glycol ointment.

recognized that the blood salicylate level at any given time represents a complex interaction of such factors as absorption, storage, biotransformation, and excretion.

Sodium salicylate was found to be absorbed through the skin to a measurable degree when incorporated in hydrophilic ointment, although the extent of absorption was considerably less than that observed with salicylic acid ointments prepared with

this base. Statistical analyses of blood salicylate concentrations indicated that there was no significant absorption of sodium salicylate from ointments compounded with petrolatum, hydrophilic petrolatum containing water, or polyethylene glycol ointment.

## DISCUSSION

**Method.**—The procedure developed in this investigation eliminates certain variables inherent in previous methods for studying percutaneous absorption, which may, in part, be responsible for the numerous contradictory statements regarding drug absorption to be found in the literature. For example, inunction of the ointment base, which is commonly employed in absorption studies, was avoided in this investigation since it is obviously difficult to control adequately the force of application and surface area involved.

Following a study of dermal permeability in chronically heat- and humidity-stressed rats, Clay and Nelson (3) suggested that the dermal permeability of drugs may be altered by stress. To minimize the occurrence of a situation which might possibly be considered as stressful, in this study all animals were conditioned to the experimental routine twice weekly for a period of one month prior to performance of the actual experiments and were not, at any time, placed in a restraining device.

Previously reported quantitative methods for studying percutaneous absorption have generally consisted of a one-point determination of test compound in either urine or blood collected at a specified interval after application of the ointment. The technique employed in this investigation, whereby blood salicylate concentration was determined at hourly intervals for an extended period of time following application of the ointment, was thought to enable a more valid comparison of the extent of percutaneous absorption of medicament from various ointment bases.

**Solubility of Drugs.**—With the ointment bases utilized in this study it was found that salicylic acid, a predominantly oil-soluble drug, was substantially absorbed through the skin and was absorbed to a much greater extent than sodium salicylate, a predominantly water-soluble compound.

It is the opinion of many investigators in this field that oil-soluble substances are absorbed through the skin, whereas water-soluble or oil-insoluble substances are not or only poorly absorbed, since the skin is largely impermeable to water and therefore to water-soluble compounds.

The percutaneous absorption of oil-soluble substances is often quite rapid and the extent of absorption appears to increase in those cases where the compound possesses a degree of water solubility in addition to oil solubility. Despite extensive reports in regard to the influence of oil and water solubility on percutaneous absorption (4-7), it has not been established that the percutaneous absorption of compounds is a function of their distribution coefficients (i. e., oil solubility/water solubility).

Bischler, *et al.* (8), refute the necessity of water solubility in regard to the absorption of compounds through the skin. They demonstrated excellent percutaneous absorption of olcates of certain alkaloids which they state to be "insoluble in water."

On the other hand, it has been shown by the use of immunologic procedures that oil-insoluble antigens are absorbed from either ointments or wet-packs (9-12), although the degree of absorption is admittedly small.

Considerable emphasis has been placed on the importance of oil solubility, since it is believed that this property tends to enhance percutaneous absorption by the transfollicular route (13). In contrast, relatively little attention has been directed to the influence of the solubility of the drug in the ointment vehicle on the extent of percutaneous absorption. This factor will be considered in a subsequent publication.

It remains equivocal as to whether sodium salicylate is absorbed through the intact skin as such or only after conversion to salicylic acid. A study by Schwenkenbecher (14) in 1904 indicated that salicylates *per se* were not absorbed percutaneously. This conclusion was drawn from an experiment in which salicylate but not lithium ion was found in a urine sample collected twenty-four hours after the topical application of a 2% lithium salicylate solution. Schwenkenbecher, therefore hypothesized that salicylate salts were converted in the skin to salicylic acid which was subsequently absorbed and excreted in the urine. More recent renal excretion studies (15) indicate, however, that the lithium ion does not appear in the urine until two to three days after the oral ingestion of lithium chloride.

Aqueous solutions of sodium salicylate (6.95%), as well as sodium salicylate ointments having a continuous aqueous phase, were found to have a pH range of 6.2 to 6.5. The pH of the skin of the rabbit was determined to range from 6.0 to 7.4, values which are in agreement with those previously reported (16). Therefore, it is doubtful that the concentration of free salicylic acid present at such pH values could account for the amounts detected in the blood of rabbits examined during this investigation.

**Effect of Type of Ointment Base.**—The results obtained in the study of percutaneous absorption of salicylic acid from various types of ointment bases, agree, in general, with the findings of Monocarp (17, 18) who demonstrated that salicylic acid was absorbed to a greater extent from an oil-in-water emulsion base than from a water-in-oil emulsion type base or an oleaginous base. These data are further substantiated by the results of a clinical study conducted by Polano, *et al.* (19), who demonstrated by a series of patch tests that the degree of skin irritation produced by 20% salicylic acid ointments was substantially greater with an oil-in-water emulsion vehicle than with a water-in-oil emulsion base. These investigators found no evidence of irritation following topical application of salicylic acid incorporated in polyethylene glycol ointment.

The influence of the ointment base on the extent of absorption of sodium salicylate was less distinct than in the case of salicylic acid, inasmuch as a measurable degree of absorption of sodium salicylate was observed only when hydrophilic ointment was used as the vehicle. Application to the rabbit's skin of sodium salicylate ointments prepared with petrolatum, hydrophilic petrolatum containing water, or polyethylene glycol ointment, was not followed by sta-

tistically significant blood salicylate levels. The possibility remains, however, that some degree of sodium salicylate absorption did occur from the latter three ointments, which could not be detected on the basis of blood salicylate level. Evidence for this assumption may be based on the known biologic properties of salicylates, and the indicative but not statistically significant blood salicylate concentrations obtained with petrolatum and hydrophilic petrolatum ointments.

Since the literature in regard to percutaneous absorption of sodium salicylate is meager, it was not possible to corroborate the results obtained in this study. However, Kionka (20) and Miyazaki (21) reported the absorption through the skin of sodium salicylate incorporated in lanolin, a water-in-oil emulsion type base, and petrolatum containing olive oil. These authors did not note any significant difference in the extent of absorption from the two types of ointment bases. Valette and Cesar (22) demonstrated the superiority of an oil-in-water type base to other liquid vehicles with respect to the percutaneous absorption of sodium salicylate.

## SUMMARY

1. A procedure, utilizing the intact rabbit skin, is described for the study of percutaneous absorption of drugs.

2. The relative extent of percutaneous absorption of salicylic acid and sodium salicylate from four different physical types of ointment bases was investigated.

3. The influence of the type of ointment base on the absorption of salicylic acid and sodium salicylate through the skin is considered.

## REFERENCES

- (1) Ungar, G., Damgaard, E., and Wong, K. W., *Proc Soc Exptl Biol Med*, 80, 45(1952).
- (2) Croxton, F. E., "Elementary Statistics with Application in Medicine," Prentice-Hall Inc., New York, N. Y., 1953.
- (3) Clay, M. M., and Nelson, J. W., *THIS JOURNAL*, 43, 230(1954).
- (4) Burgi, E., "Die Durchlässigkeit der Haut für Arzneien und Gifte," Springer, Berlin, Germany, 1942, p. 110.
- (5) Rothman, S., *J Lab Clin Med*, 28, 1305(1943).
- (6) Calvery, H. O., Draize, J. H., and Laug, E. P., *Physiol Rev*, 26, 495(1946).
- (7) Valette, G., and Caver, R., *J Physiol*, 39, 137(1917).
- (8) Bischler, A., Favre, M., Frommel, E., and Valette, G., *Acta Pharmacol Toxicol*, 4, 122(1948).
- (9) Kimura, G., *Orient J Dis Infants*, 28, 15(1940).
- (10) Walzer, A., and Sack, S. S., *Arch Dermatol and Syphilol*, 49, 427(1944).
- (11) Walzer, A., *ibid*, 41, 692(1940).
- (12) Golovanoff, M., *Compt rend soc biol*, 94, 6(1926).
- (13) Rothman, S., *J Soc Cosmetic Chemists*, 6, 193(1955).
- (14) Schwenkenbecher, A., *Arch Anat u Physiol*, 121, 65(1904).
- (15) Foulks, J., Mudge, G. H., and Gilman, A., *Am J Physiol*, 168, 642(1952).
- (16) Draize, J. H., *J Invest Dermatol*, 5, 77(1912).
- (17) Monocarp, C., *Arch exptl Pathol Pharmacol*, 141, 50(1929).
- (18) Monocarp, C., *ibid*, 141, 87(1929).
- (19) Polano, M. K., Bonzel, J., and Van Der Meer, B. J., *Dermatologica*, 101, 69(1950).
- (20) Kionka, H., *Klin Wochschr*, 10, 1570(1931).
- (21) Miyazaki, K., *Japan J. Dermatol. Urol.*, 31, 113(1931).
- (22) Valette, G., and Cesar, R., *Ann. pharm. franc*, 6, 16(1948).

# The Effect of Various Ointment Bases on the Percutaneous Absorption of Salicylates II\*

## Effect of Surface-Active Agents

By MOISE E. STOLAR†, G. VICTOR ROSSI, and MARTIN BARR

The effect of the inclusion of several concentrations of surface-active agents in four different types of ointment bases on the percutaneous absorption of salicylic acid and sodium salicylate was determined. Hydrophilic ointment, petrolatum, hydrophilic petrolatum containing water, and polyethylene glycol ointment were modified either by varying the concentration of surface-active agent present in the U. S. P. XV formula, or by adding varying amounts of surface-active agents to those bases in which none was originally present. Sorbitan monostearate, polyoxyethylene 20 sorbitan monostearate, and polyoxyethylene 40 stearate were studied as representative of lipophilic and hydrophilic surface-active agents of the nonionic type. The results indicate a correlation between percutaneous absorption and the degree of interaction of the polyoxyethylene groups present in the hydrophilic surface-active agents with the salicylates.

THE CURRENT CONCEPT of the role played by the ointment base in percutaneous absorption is that it facilitates contact between the medicament and the absorbing cells of the sebaceous glands and hair follicles. Bliss (1) has indicated that the properties of the drug itself, rather than the ointment vehicle, are the major determining factors in absorption from the skin. The vehicle may, however, modify the absorption properties of drugs which are capable of penetrating the skin.

Surface-active agents provide a means of improving ointment bases and enhancing the percutaneous absorption of drugs, as indicated by Dodd, *et al.* (2), by promoting diffusion of the medicament from the vehicle, or as suggested by Sweet (3), by emulsifying the sebum.

Although it is the opinion of several investigators (2-5) that surface-active agents increase the absorption of compounds incorporated in topical preparations, there are relatively little *in vivo* experimental data to substantiate this hypothesis. The present study was instituted to provide additional information in reference to the influence of surface-active agents on the absorption of drugs from ointment bases. The factors specifically considered were the effects of several surfactants, incorporated in various concentrations in four different physical types of ointment bases, on the percutaneous absorption of salicylic acid, a primarily oil-soluble drug,

and sodium salicylate, a predominantly water-soluble compound.

Petrolatum U. S. P. XV; hydrophilic petrolatum U. S. P. XV, to which water was added; hydrophilic ointment U. S. P. XV; and polyethylene glycol ointment U. S. P. XV were modified either by varying the concentration of surface-active agent indicated in the U. S. P. formula, or by adding various concentrations of different surfactants to those bases in which none was originally present. Sorbitan monostearate (Span 60),<sup>1</sup> polyoxyethylene 20 sorbitan monostearate (Tween 60),<sup>1</sup> and polyoxyethylene 40 stearate (Myrj 52)<sup>1</sup> were selected as representative of lipophilic and hydrophilic surface-active agents of the nonionic type.

In order to elucidate the results obtained with ointments prepared from bases containing polyethylene glycols or surface-active agents of the polyoxyethylene sorbitan fatty acid ester type or the polyoxyethylene esters of fatty acid type, the effect of such substances on the solubility of salicylic acid and sodium salicylate in aqueous solutions was studied.

## EXPERIMENTAL

The experimental procedure and methods of analysis for salicylic acid and sodium salicylate in the ointment bases and in blood were previously described (6).

**Preparation of Ointment Bases.**—The various modifications of petrolatum and polyethylene glycol ointment were prepared by adding specified amounts (see Table I) of either Span 60 or Tween 60 to these bases warmed to 70°. The 70° temperature was maintained until the surface-active agents were either dissolved or dispersed in the melted base which was then allowed to cool to room temperature with constant stirring.

\* Received July 16, 1959, from the Philadelphia College of Pharmacy and Science, Philadelphia 4, Pa.

Abstracted from a thesis submitted by Moise E. Stolar to the faculty of the Philadelphia College of Pharmacy and Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: Warner Lambert Research Institute, Morris Plains, N. J.

The authors wish to thank Chas. Pfizer and Co. for their financial support of this study.

<sup>1</sup> Atlas Powder Co., Wilmington, Del.

SCIENTIFIC EDITION

TABLE I.—CONCENTRATION OF SURFACE-ACTIVE AGENTS PRESENT IN OINTMENT BASES

Surface-Active Agent	Petrolatum	Hydrophilic Petrolatum <sup>a</sup>	Hydrophilic Ointment	Polyethylene Glycol Ointment
Sorbitan monostearate	1, 5, 10%	1, 5, 10% <sup>b</sup>	3, 5, 10% <sup>c</sup>	1, 5, 10%
Polyoxyethylene 20 sorbitan monostearate	1, 5, 10%		3, 5, 10%	1, 5, 10%
Polyoxyethylene 40 stearate				...

<sup>a</sup> 15 Parts purified water added to 100 parts of base  
<sup>b</sup> Cholesterol omitted from U. S. P. XV formula  
<sup>c</sup> Polyoxyethylene 40 stearate omitted from U. S. P. XV formula

The modifications of hydrophilic ointment and hydrophilic petrolatum, incorporating various concentrations of surfactants, were prepared essentially in accordance with the directions given in the corresponding U. S. P. XV monographs. To 100 parts of hydrophilic petrolatum, and each of its variations, were added 15 parts of purified water, warmed to 72°.

Salicylic acid ointments containing 6% of the active ingredient, and sodium salicylate ointments containing 6.95% of the sodium derivative (equivalent to 6% salicylic acid) were prepared in the manner previously described (6).

Solubility Studies.—The solubilities of salicylic acid and sodium salicylate in aqueous solutions containing varying amounts of polyethylene glycol 400, Tween 60, and Myrj 52, were determined. A spectrophotometric method was used for all analytical determinations (7).

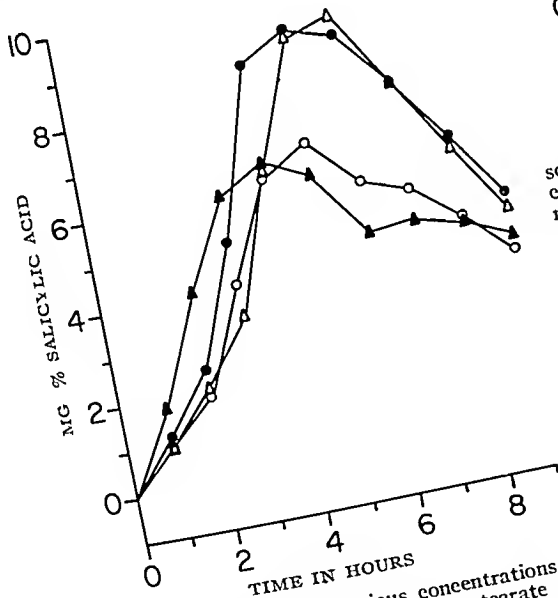


Fig. 1.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in petrolatum on the percutaneous absorption of salicylic acid. ▲, petrolatum; polyoxyethylene 20 sorbitan monostearate: O, 1%; Δ, 5%; ●, 10%.

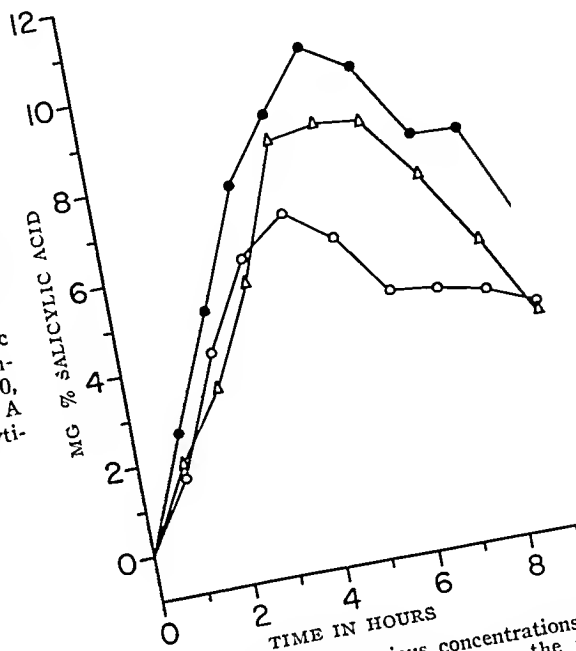


Fig. 2.—The effect of various concentrations of sorbitan monostearate in petrolatum on the percutaneous absorption of salicylic acid. Sorbitan monostearate: O, 1%; Δ, 5%; ●, 10%.

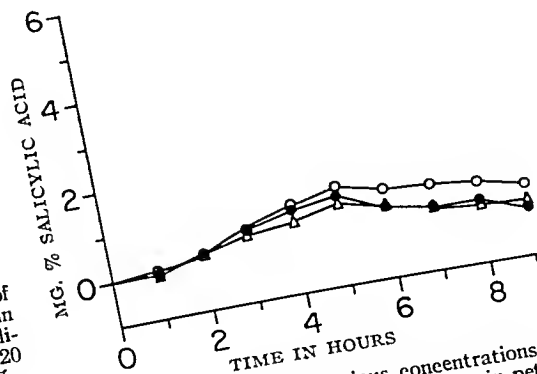


Fig. 3.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in petrolatum on the percutaneous absorption of sodium salicylate. Polyoxyethylene 20 sorbitan monostearate: O, 1%; Δ, 5%; ●, 10%.

RESULTS

The results obtained in this investigation indicate that the presence of surface-active agents in ointment bases may increase, decrease, or have no effect

on the extent of absorption of salicylic acid and sodium salicylate through the intact rabbit skin, depending primarily on the properties of the surfactant and the ointment base.

The addition of Tween 60 or Span 60 to petrolatum had no effect on the extent of percutaneous absorption of salicylic acid when these surfactants were present in a concentration of 1%, as may be seen in Figs. 1 and 2. A marked increase in drug absorption occurred in all cases when the concentration of these surfactants was increased to 5 or 10%, however, there was no significant difference either between the effects produced by the two different

surface-active agents or between the 5 and 10% concentrations.

Addition of either 1, 5, or 10% of Tween 60 to petrolatum had no effect on the percutaneous absorption of sodium salicylate (Fig. 3). The incorporation of Span 60 in concentrations of 1, 5, and 10% in petrolatum, in each case, resulted in a marked increase in the extent of sodium salicylate absorption (Fig. 4), however there was no significant difference among the effects produced by the different concentrations.

Span 60 incorporated in 5 or 10% concentrations in hydrophilic petrolatum, to which water was added, did not alter the extent of percutaneous absorption of salicylic acid or sodium salicylate as compared to the absorption of these compounds from the base without the inclusion of the surfactant (Figs. 5 and 6). A decrease in the absorption of salicylic acid was observed upon the addition of increasing concentrations of Tween 60 (Fig. 7) and Myrj 52 (Fig. 8) to hydrophilic ointment, however, the differences in blood salicylate levels obtained with the 3 and 5% concentrations of both surfactants were not statistically significant. There was no comparable reduction in the extent of absorption when these bases were employed as vehicles for sodium salicylate (Figs. 9 and 10).

On the basis of the blood salicylate level, neither salicylic acid nor sodium salicylate was absorbed to a significant extent through the skin of the rabbit when these compounds were incorporated in polyethylene glycol ointment (6). Furthermore, the inclusion of various concentrations of either Myrj 52 or Tween 60 did not lead to a significant degree of absorption of salicylic acid or sodium salicylate from polyethylene glycol ointment (7).

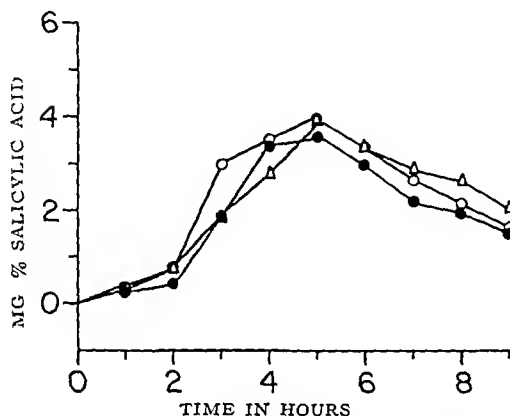


Fig. 4.—The effect of various concentrations of sorbitan monostearate in petrolatum on the percutaneous absorption of sodium salicylate. Sorbitan monostearate: O, 1%; Δ, 5%; ●, 10%.

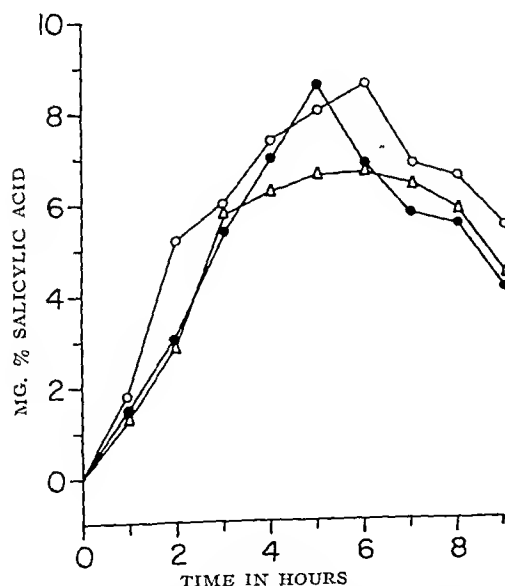


Fig. 5.—The effect of various concentrations of sorbitan monostearate in hydrophilic petrolatum containing water on the percutaneous absorption of salicylic acid. Hydrophilic petrolatum containing water, O; sorbitan monostearate: Δ, 5%; ●, 10%.

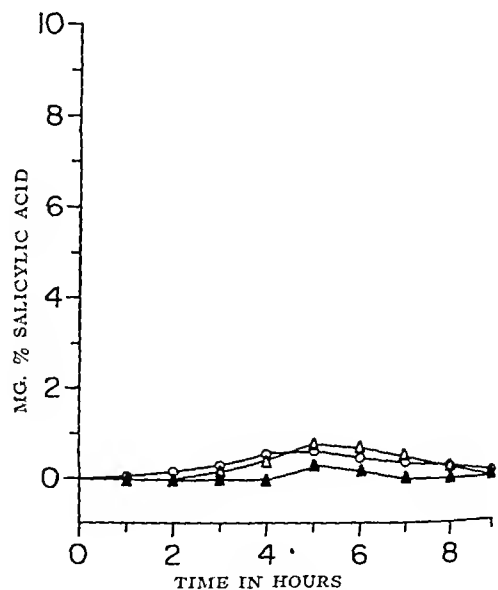


Fig. 6.—The effect of various concentrations of sorbitan monostearate in hydrophilic petrolatum containing water on the percutaneous absorption of sodium salicylate. Hydrophilic petrolatum containing water, Δ; sorbitan monostearate: O, 5%; Δ, 10%.

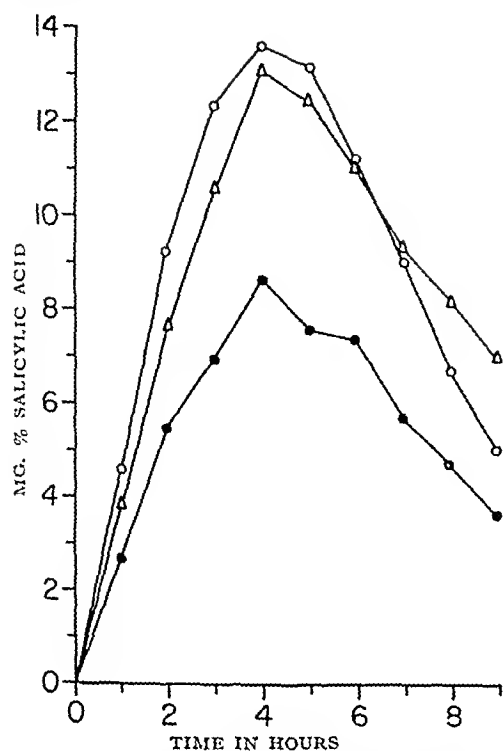


Fig. 7.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in hydrophilic ointment on the percutaneous absorption of salicylic acid. Polyoxyethylene 20 sorbitan monostearate: O, 3%; Δ, 5%; ●, 10%.

### DISCUSSION

One of the most interesting observations made during this study was that when substances containing polyoxyethylene groups were present in ointment bases, a marked reduction in the extent of percutaneous absorption of salicylic acid resulted. This was demonstrated in studies performed with hydrophilic ointment containing Tween 60 and Myrj 52, and in the experiments utilizing polyethylene glycols.

Numerous papers have noted the interaction of compounds possessing phenolic hydroxyl groups with substances containing polyoxyethylenic groups (8-14). Salicylic acid, in particular, has been shown to interact with polyoxyethylene groups (13). Therefore, it may be assumed that the reduction in the absorption of salicylic acid from ointment bases containing substances having polyoxyethylene groups is due to an interaction between these groups and the phenolic hydroxyl group of salicylic acid. The degree of interaction was assessed by determining the aqueous solubilities of salicylic acid and sodium salicylate in the presence of various concentrations of polyethylene glycol 400, Tween 60, and Myrj 52.

It was found that an increase in the concentration of the surface-active agents containing polyoxyethylenic groups increased the aqueous solubility of salicylic acid (Table II). In addition, the solubility of salicylic acid increased progressively with an increase in the concentration of polyethylene glycol

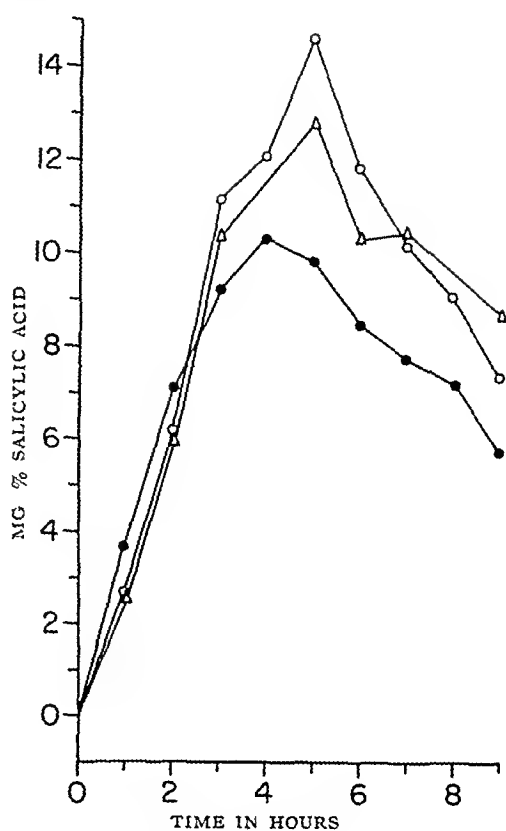


Fig. 8.—The effect of various concentrations of polyoxyethylene 40 stearate in hydrophilic ointment on the percutaneous absorption of salicylic acid. Polyoxyethylene 40 stearate: O, 3%; Δ, 5%; ●, 10%.

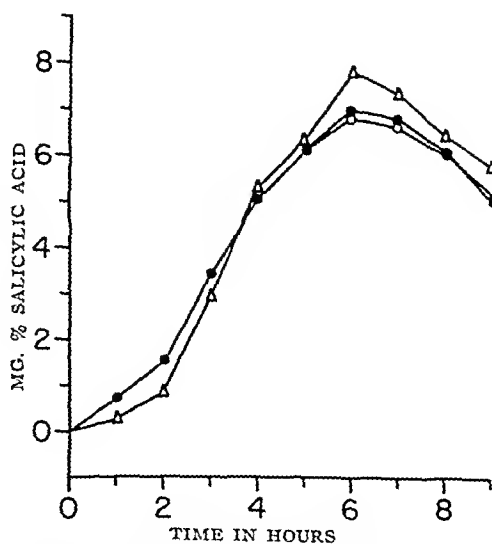


Fig. 9.—The effect of various concentrations of polyoxyethylene 20 sorbitan monostearate in hydrophilic ointment on the percutaneous absorption of sodium salicylate. Polyoxyethylene 20 sorbitan monostearate: O, 3%; Δ, 5%; ●, 10%.



TABLE II.—SOLUBILITY (25°) OF SALICYLIC ACID IN WATER<sup>a</sup> CONTAINING VARIOUS CONCENTRATIONS OF POLYETHYLENE GLYCOL 400, Tween 60, or Myrj 52

Polyethylene Glycol 400, % w/v	Salicylic Acid in Solution, Gm/100 ml	Tween 60, % w/v	Salicylic Acid in Solution, Gm/100 ml	Myrj 52, % w/v	Salicylic Acid in Solution, Gm/100 ml
0	0.23	0	0.23	0	0.23
1	0.25	1	0.37	1	0.37
3	0.27	3	0.61	3	0.60
6	0.31	5	0.86	5	0.87
10	0.41	7	1.06	7	0.94
15	0.49	10	1.47	10	1.43
20	0.60				
30	1.16				
40	2.10				
50	6.67				

<sup>a</sup> Sulfuric acid added to all solutions to produce a final concentration of 0.003 N H<sub>2</sub>SO<sub>4</sub>.

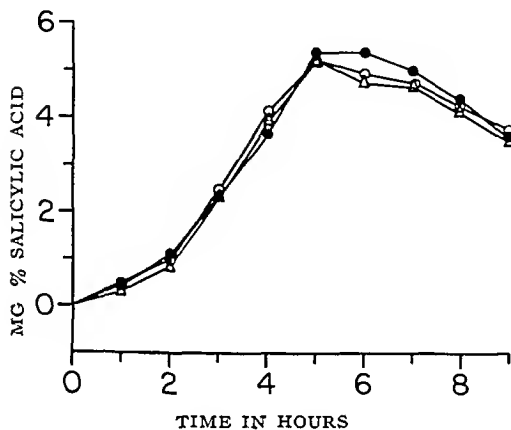


Fig. 10.—The effect of various concentrations of polyoxyethylene 40 stearate in hydrophilic ointment on the percutaneous absorption of sodium salicylate. Polyoxyethylene 40 stearate: O, 3%; Δ, 5%; ●, 10%.

400 in the aqueous medium, whereas the solubility of sodium salicylate remained almost unchanged in the presence of substances containing polyoxyethylene groups (7).

Various explanations have been advanced to account for the improved solubility of substances in the presence of nonionic surfactants containing polyoxyethylene groups (15, 16). Regardless of the mechanism involved in the interaction of salicylic acid and the surface-active agents, from the results of this study, it appears that a relationship may exist between the percutaneous absorption of salicylic acid and its tendency to associate with polyoxyethylene groups.

Although the solubility of salicylates in sebum was not determined in this study, it may be theorized that one of the factors influencing the percutaneous absorption of a drug is its solubility in the vehicle as compared to its solubility in sebum. A distribution coefficient principle may be operative, i. e., the greater the solubility of the drug in the vehicle as compared to the sebum, the less the absorption through the intact skin.

The aqueous solubility of salicylic acid was found

to be improved in the presence of compounds containing polyoxyethylene groups. Since emulsion bases contain water either as a continuous or as a dispersed phase, and since they also contain surfactants, the solubility of the drugs dispersed in such bases varies depending upon the specific surface-active agent present and its concentration. Such differential solubility, may, at least partly, account for the results obtained in this investigation as well as the results reported by other investigators.

## SUMMARY

1. The effect of the presence of various surface-active agents in petrolatum, hydrophilic petrolatum, hydrophilic ointment, and polyethylene glycol ointment, on the absorption of salicylic acid and sodium salicylate through the intact rabbit skin was investigated.

2. A concept is presented which relates the extent of percutaneous absorption of a drug to its relative solubility in sebum and the vehicle in which it is incorporated

## REFERENCES

- (1) Bliss, A. R., *THIS JOURNAL*, 25, 694 (1936)
- (2) Dodd, M. C., Hartmann, F. W., and Ward, W. C., *ibid.*, 35, 33 (1946)
- (3) Sweet, D. R., *Practitioner*, 167, 53 (1951)
- (4) Laug, E. P., Vos, E. A., Umberger, E. J., and Kunze, F. M., *J. Pharmacol. Exptl. Therap.*, 89, 42 (1947)
- (5) Duemling, W. W., *Arch. Dermatol. and Syphilol.*, 43, 264 (1941)
- (6) Stolar, M. E., Rossi, G. V., and Barr, M., *THIS JOURNAL*, 49, 144 (1960)
- (7) Stolar, M. E., "The Effect of Various Ointment Bases on the Percutaneous Absorption of Salicylates Through the Intact Rabbit Skin," Ph. D. Thesis, 1959, Philadelphia College of Pharmacy and Science
- (8) Guttman, D., and Higuchi, T., *THIS JOURNAL*, 45, 659 (1956)
- (9) Plaxco, J. M., and Husa, W. J., *ibid.*, 45, 141 (1956)
- (10) Bolle, A., and Mirmanoff, A., *J. Pharm. and Pharmacol.*, 2, 685 (1950)
- (11) Bouchardy, M., and Mirmanoff, A., *Pharm. Acta Helv.*, 26, 284 (1951)
- (12) Lawrence, C. A., and Erlanson, A. L., *THIS JOURNAL*, 42, 352 (1953)
- (13) Higuchi, T., and Lach, J. L., *ibid.*, 43, 465 (1954)
- (14) Chakravarty, D., Lach, J. L., and Blaug, S. M., *Drug Standards*, 25, 137 (1957)
- (15) Patel, N. K., and Kostenbader, H. B., *THIS JOURNAL*, 47, 289 (1958)
- (16) Allawala, N. A., and Riegelman, S., *ibid.*, 42, 396 (1953).

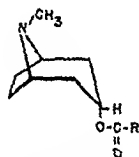
# The Synthesis of Some Glycidic Esters as Potential Antispasmodics\*

By GARY OMODT† and OLE GISVOLD

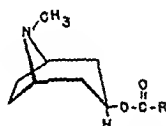
When tropine is esterified with an appropriate acid, an active antispasmodic results. In order to study a change in the linkage between the nitrogen ring system and the acid moiety, a series of glycidic esters was prepared utilizing tropinone as the starting ketone in the Darzens condensation. Another series was prepared utilizing 1-methyl-4-piperidone.

PREVIOUS PUBLICATIONS (1-5) indicate that tropine is, in general, more active as antispasmodics than pseudotropine. If a Fisher-Taylor-Hirschfelder model of a tropine is compared to that of a pseudotropine, it can be seen that the distance between the N-methyl and the carbonyl of the tropine differs significantly from the corresponding distance in the pseudotropine. Presumably, the N-methyl-carbonyl distance in a tropine is more favorable for competition with acetylcholine than is the N-methyl-carbonyl distance in a pseudotropine. This could account for the difference in activity.

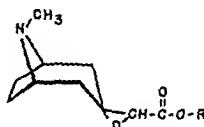
If tropinone is condensed with ethyl chloroacetate and the product transesterified with an appropriate alcohol, a compound will be obtained that would structurally resemble both a tropine and a pseudotropine and yet would possess an N-methyl-carbonyl distance differing from both. The purpose of this study is to determine if this would be beneficial or detrimental to antispasmodic activity.



A TROPINE



A PSEUDOTROPINE



A GLYCIDIC ESTER

It can be seen that the conformation of the epoxide ring would affect the N-methyl-carbonyl

\* Received August 17, 1959, from the College of Pharmacy, University of Minnesota, Minneapolis 14.

Abstracted from a thesis submitted to the Graduate Faculty of the University of Minnesota by Gary Omodt in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Present address: South Dakota State College of Agriculture and Mechanic Arts, Brookings, S. D.

distance and thus the activity of the compound. Nevertheless, no attempt was made to determine the isomeric content of the compounds prepared.

A preliminary pharmacological evaluation indicated that this type of glycidic ester possessed considerable antispasmodic activity (see Fig. 1).

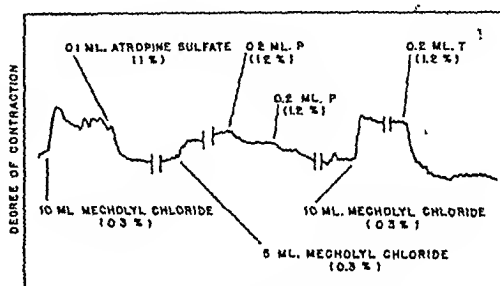


Fig. 1.—Pharmacological evaluation of benzohydryl 6-methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate hydrochloride (P) and benzohydryl 5,8-methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate hydrochloride (T).

The alcohols that were used in the transesterifications were: benzohydrol, dicyclohexylmethanol, cyclohexanol, and 1-methyl-4-piperidinol. Benzohydrol was chosen in an attempt to simulate the configuration of diphenylacetyl tropine (an active antispasmodic). Cyclohexanol was used to determine the effect of size of the alkyl portion on the activity, and 1-methyl-4-piperidinol (assuming the cyclohexyl esters to be active) was chosen to see what effect a basic nitrogen, located equidistant on each side of the carbonyl, would have on activity. Quaternization of the product, obtained when transesterifying with this last alcohol, should result in a compound possessing ganglionic blockade activity. The hydrochlorides of the benzohydryl glycidic esters were extremely sensitive to hydrolysis, probably due to the stability of the benzohydryl carbonium ion. An attempt was made to reduce this rapid hydrolysis rate by substituting the dicyclohexylmethyl group in place of the benzohydryl group. This would remove the possibility of formation of such a stable carbonium ion.

## METHODS OF PREPARATION

Tropinone was prepared according to the procedure of Keagle, *et al.* (6). In the preparation of 1-methyl-4-piperidone, the method of McElvain, *et al.* (7), was used.

The Darzens condensation for the synthesis of glycidic esters employs a basic catalyst. The most commonly used catalysts are sodium ethoxide or sodamide (8), and in some cases potassium tertiary butoxide (9). Sodium hydride recently has been reported to be a useful catalyst in the Darzens condensation (10). Because sodium hydride-mineral oil suspension is so easily handled and stored, it was used as the Darzens catalyst. A general laboratory method for running a Darzens condensation was obtained from *Organic Reactions* (11).

A search of the literature indicated that dicyclohexylmethanol had been prepared by Mousseron, *et al.* (12), and also by Sabatier, *et al.* (13), via the Grignard reaction. However, the reaction conditions were not described in detail by either worker so the method of synthesis of di-*n*-butyl carbinol in *Organic Syntheses* (14) was followed.

1-Methyl-4-piperidinol could easily be produced by catalytic reduction of 1-methyl-4-piperidone in the Parr bomb (15) but an alternate method was utilized because of the excellent yields and the simplicity of reaction. This was the sodium borohydride reduction of 1-methyl-4-piperidone as described by Counsel (16) for the preparation of 2-hydroxyquinolizidine.

The syntheses of the different alkyl glycidic esters were carried out by following the method of transesterification as modified by Counsel (17). This method makes use of sodium hydride as the catalyst and was quite applicable to all of the glycidic ester syntheses attempted. The general procedure consisted of dissolving the appropriate alcohol and the ethyl glycidic ester in 100-150 ml. of anhydrous, redistilled Skellysolve C. The solution in a 500-ml., three-necked, round-bottomed flask equipped with a stirrer, Dean-Stark separator, reflux condenser, and drying tube was stirred and refluxed. A catalytic amount of sodium hydride suspension in mineral oil then was added to the refluxing solution. At this point, the reaction mixture became turbid and yellow colored. Reflux was continued, with stirring, for twenty hours, after which the Skellysolve C was removed by distillation under reduced pressure. When 15-20 ml. of material remained in the distilling flask, 200 ml. of ether was added and the resulting solution was washed four times with 50-ml. portions of distilled water. The washed ether solution was dried over anhydrous sodium sulfate for thirty minutes in the refrigerator. The ether was then removed by distillation under reduced pressure and the residue distilled *in vacuo*.

## BIOLOGICAL RESULTS

The pharmacological evaluation of the first two glycidic esters that were prepared was attempted in order to obtain some indication as to whether the glycidic esters of the type described might possess some antispasmodic activity. Since quite positive results were obtained (see Fig. 1), other potentially

active esters were synthesized. It should be admitted, however, that only one evaluation was run on each compound and this cannot be taken as good pharmacological evidence.

## EXPERIMENTAL

All melting points recorded in this paper were taken by using a capillary tube melting point apparatus. The melting points are all uncorrected. The elemental analyses were performed by the microanalytical laboratory at the Department of Chemistry, University of Minnesota.

**Ethyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate (I) and Its Picrate.**—A solution of 48.8 Gm. (0.43 mole) of 1-methyl-4-piperidone and 53 Gm. (0.43 mole) of ethyl chloroacetate in 350 ml. of anhydrous toluene was placed in a one-liter, three-necked, round-bottomed flask equipped with a stirrer, thermometer, reflux condenser, and drying tube. The flask previously had been dried by heating with a flame while being swept out with a stream of dry nitrogen. The entire reaction was carried out under a stream of dry nitrogen. The reactants were cooled to 5° and 30.9 Gm. (0.69 mole) of a 53.7% suspension of sodium hydride in mineral oil was added as rapidly as possible while maintaining the temperature at 5° with a dry ice-acetone bath. After the addition, commercial absolute ethanol (50 drops) was added, dropwise, to the stirred mixture while the temperature was maintained at 5°. After the effervescence had subsided, the mixture was allowed to warm to room temperature and was stirred overnight. Glacial acetic acid (41.5 Gm., 0.69 mole) was then added while stirring and cooling in an ice bath. Enough water was added to dissolve the sodium acetate and the aqueous layer was removed and neutralized in the cold with potassium carbonate. The toluene layer was extracted with six 100-ml. portions of cold 3 *N* hydrochloric acid. Each portion was neutralized immediately with potassium carbonate in the cold. All of the extracts (including the neutralized acetic acid portion) were mixed and salted out with potassium carbonate and extracted continuously with ether for three days. After drying the ether extract over anhydrous sodium sulfate for one day and removal of the ether by distillation, the residue was vacuum distilled. The yellow colored product, distilling at 85-100°/0.35 mm., weighed 61.4 Gm. (72.3% yield);  $n_D^{25}$  1.4650.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of ethanol and adding dropwise with stirring, a saturated solution of picric acid in ethanol. The picrate was recrystallized four times from an ethanol-water mixture (1:1) and dried overnight at 100° under vacuum; m.p. 174-175°.

*Anal.*—Calcd. for  $C_{10}H_{17}NO_3 \cdot C_6H_3N_3O_7$ : C, 44.86; H, 4.79. Found; C, 44.85; H, 4.71.

**Ethyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate (II) and Its Picrate.**—This compound was prepared by following the method of synthesis of compound I with the following modifications. Tropinone (37.6 Gm., 0.27 mole) was mixed with 33.2 Gm. (0.27 mole) of ethyl chloroacetate in 350 ml. of anhydrous toluene. The reactants were cooled to -10° with a dry ice-acetone bath and 19.3 Gm. (0.43 mole) of a 53.7% suspension of sodium hydride in mineral oil was

added as rapidly as possible. After the addition, commercial absolute ethanol (60 drops) was added dropwise to the stirred reaction mixture while maintaining the temperature at  $-10^{\circ}$ . The temperature then was allowed to rise slowly while keeping the effervescence under control by raising and lowering the cooling bath. After the effervescence had subsided, the mixture was allowed to warm to room temperature and stirring was maintained for eighteen hours. Glacial acetic acid (25.9 Gm., 0.43 mole) then was added to the stirred suspension while cooling in an ice bath. The yellow colored product, distilling at  $110-130^{\circ}/0.3$  mm., weighed 28.3 Gm. (46.5% yield);  $n_D^{25}$  1.4850.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The picrate was recrystallized four times from ethanol and dried overnight at  $100^{\circ}$  under vacuum; m.p.  $154-155^{\circ}$ .

*Anal.*—Calcd. for  $C_{17}H_{19}NO_3 \cdot C_6H_3N_3O_7$ : C, 47.58; H, 4.88. Found: C, 47.65; H, 5.01.

**Dicyclohexylmethanol.**—Into a three-liter, three-necked, round-bottomed flask fitted with a 500-ml. dropping funnel, a stirrer, and a reflux condenser with drying tube, was placed 36.5 Gm. (1.5 gram-atoms) of magnesium turnings and 500 ml. of absolute ether. The synthesis was carried out by following the method for the synthesis of di-*n*-butyl carbinol in *Organic Synthesis* (14) and using correct molecular proportions of additional reagents. Also, the reaction mixture was not steam distilled. The cooled mixture of carbinol and aqueous potassium hydroxide was extracted first with 200 ml. of ether and then with three additional 100-ml. portions of ether. The combined ether extracts were dried over anhydrous potassium carbonate for one hour. After removal of the ether by distillation under reduced pressure, the residue was distilled, *in vacuo*, through a fractionating column, the fraction distilling at  $125-128^{\circ}/4.5$  mm. being the product [reported b.p.  $166^{\circ}/20$  mm. (13)]. The yield was 89.3 Gm. (60.8%).

**1-Methyl-4-piperidinol.**—A solution of 1-methyl-4-piperidone (56.5 Gm., 0.5 mole) in 200 ml. of water was added slowly to a solution of 5.5 Gm. of sodium borohydride in 200 ml. of water. The mixture was stirred and kept cool with an ice bath during the addition. After allowing the mixture to stand at room temperature overnight, 73 ml. of concentrated ammonium hydroxide solution was added and the mixture allowed to stand for an additional hour. The solution then was saturated with anhydrous sodium sulfate. Two layers formed. The upper layer was removed and the lower layer was extracted two times with 200-ml. portions of hot benzene. The extracts were combined with the upper layer and the solvents were removed by distillation under reduced pressure. The residue was vacuum distilled, the product distilling at  $85-88^{\circ}/6$  mm. [reported b. p.  $83^{\circ}$  under 8 mm. (18)]. The yield was 40.5 Gm. (70.4%);  $n_D^{25}$  1.4770.

**Benzohydril 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its Hydrochloride and Picrate.**—This compound was prepared by slightly modifying the general procedure for transesterification. Benzohydrol (5.2 Gm., 0.028 mole) and 5.6 Gm. (0.028 mole) of I were dissolved in 100 ml. of

anhydrous, redistilled Skellysolve C. After bringing the solution to reflux temperature and addition of the catalyst, the first ten 10-ml. portions of distillate were removed, each with the simultaneous addition of a 10-ml. portion of fresh Skellysolve C to the reaction flask. The reaction mixture was then refluxed for twenty hours and worked up in the usual manner. The yellow colored product, distilling at  $200-205^{\circ}/0.4$  mm., weighed 4.4 Gm. (46.7% yield);  $n_D^{25}$  1.5525.

A hydrochloride was prepared by adding, dropwise with stirring, an ethereal solution of hydrogen chloride to a solution of 1 Gm. of material dissolved in 25 ml. of absolute ether. The precipitated hydrochloride was filtered under an atmosphere of dry nitrogen because it was so hygroscopic. It was recrystallized once from a mixture of commercial absolute ethanol and isopropyl ether. Further attempts at recrystallization for the purpose of preparing an analytical sample resulted in an oil which could not be recrystallized. The oil was forced to crystallize by drying under high vacuum at  $100^{\circ}$ ; m.p.  $173-175^{\circ}$  (decompn.). This material was submitted, as such, for analysis.

*Anal.*—Calcd. for  $C_{21}H_{23}NO_3 \cdot HCl$ : C, 67.46; H, 6.47. Found: C, 59.11; H, 6.28.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from an ethanol-water mixture (3:1) and dried overnight at  $100^{\circ}$  under vacuum; m. p.  $189-190^{\circ}$ .

*Anal.*—Calcd. for  $C_{21}H_{23}NO_3 \cdot C_6H_3N_3O_7$ : C, 57.24; H, 4.63. Found: C, 57.33; H, 4.70.

**Dicyclohexylmethyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its *p*-Toluene Sulfonate and Picrate.**—Anhydrous dicyclohexylmethanol (5.9 Gm., 0.03 mole) and 5.9 Gm. (0.03 mole) of I were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.<sup>1</sup> The yellow colored product, distilling at  $170-188^{\circ}/0.4$  mm., weighed 7.2 Gm. (68.8% yield);  $n_D^{25}$  1.4923.

A *p*-toluene sulfonate was prepared by dissolving 1 Gm. of material in 25 ml. of absolute ether and adding, dropwise with stirring, a saturated solution of *p*-toluene sulfonic acid monohydrate in absolute ether. The precipitated *p*-toluene sulfonate was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at  $100^{\circ}$  under vacuum; m. p.  $175-176^{\circ}$ .

*Anal.*—Calcd. for  $C_{21}H_{23}NO_3 \cdot C_7H_7O_2S$ : C, 64.46; H, 8.31. Found: C, 64.67; H, 8.33.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from commercial absolute ethanol and dried overnight at  $100^{\circ}$  under vacuum; m. p.  $191-192^{\circ}$  (decompn.).

*Anal.*—Calcd. for  $C_{21}H_{23}NO_3 \cdot C_6H_3N_3O_7$ : C, 56.04; H, 6.62. Found: C, 55.49; H, 6.60.

<sup>1</sup> Modified by packing with glass wool and calcium hydride so as to prevent any reflux of ethanol into the reaction mixture.

**Cyclohexyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its *p*-Toluene Sulfonate and Picrate.**—Anhydrous cyclohexanol (12.8 Gm., 0.128 mole) and 7.2 Gm. (0.064 mole) of I were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.<sup>1</sup> The yellow colored product, distilling at 145–155°/0.2 mm., weighed 6.3 Gm. (37.2% yield);  $n_D^{25}$  1.4828.

A *p*-toluene sulfonate was prepared by dissolving 1 Gm. of material in 25 ml. of absolute ether and adding, dropwise with stirring, a saturated solution of *p*-toluene sulfonic acid monohydrate in anhydrous ether. The precipitated *p*-toluene sulfonate was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 132–133°.

*Anal.*—Calcd. for  $C_{14}H_{23}NO_3 \cdot C_7H_9O_3S$ : C, 59.27; H, 7.34. Found: C, 59.35; H, 7.34.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The oily picrate was frozen in a dry ice-acetone bath and crushed with a stirring rod. The powdered picrate was recrystallized four times from methanol and dried overnight at 100° under vacuum; m. p. 139–140°.

*Anal.*—Calcd. for  $C_{14}H_{23}NO_3 \cdot C_6H_3N_3O_7$ : C, 49.79; H, 5.43. Found: C, 49.83; H, 5.51.

**1-Methyl-4-piperidyl 6-Methyl-1-oxa-6-aza-spiro-(2,5)-octane-2-carboxylate and Its Methyl Bromide and Picrate.**—This compound was prepared by slightly modifying the general procedure for transesterification. Anhydrous 1-methyl-4-piperidinol (5.8 Gm., 0.05 mole) and 10 Gm. (0.05 mole) of I were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C. After bringing the solution to reflux temperature and addition of the catalyst, the first 25 ml. of distillate were removed and reflux was continued, with stirring, for eighteen hours, after which, the Skellysolve C was removed by distillation under reduced pressure. The residue was distilled *in vacuo*.<sup>2</sup> The yellow colored product, distilling at 145–155°/1 mm., weighed 6.7 Gm. (50% yield);  $n_D^{25}$  1.4876.

A methyl bromide was prepared by dissolving 1 Gm. of material in 20 ml. of anhydrous ether and adding 20 ml. of methyl bromide. The mixture was kept for three days at room temperature in a pressure bottle. The precipitated methyl bromide was filtered under dry nitrogen and recrystallized four times from a methanol-isopropyl ether mixture (filtering under dry nitrogen each time because of hygroscopicity). The hygroscopic methyl bromide was dried overnight at 100° under vacuum. A melting point could not be obtained under 270°. The compound, however, decomposed with charring over the range 189–270°.

*Anal.*—Calcd. for  $C_{14}H_{21}N_2O_3 \cdot 2CH_3Br \cdot \frac{1}{2}H_2O$ : C, 41.12; H, 6.69. Found: C, 40.99; H, 6.92.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding it, dropwise with stirring, to 20 ml. of a saturated solution of picric acid in commercial

absolute ethanol. The precipitated picrate was recrystallized four times from an acetone-ethanol mixture. The picrate first was dissolved in a minimum amount of acetone and an equal volume of commercial absolute ethanol was added to it. The resulting solution then was concentrated to one-half of its original volume and crystallization was allowed to take place. The recrystallized picrate was dried overnight at 100° under vacuum; m. p. 203–204° (decompn.).

*Anal.*—Calcd. for  $C_{14}H_{21}N_2O_3 \cdot 2C_6H_3N_3O_7$ : C, 42.98; H, 4.16. Found: C, 42.89; H, 4.35.

**Benzohydryl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its Hydrochloride and Picrate.**—Benzohydrol (10.3 Gm., 0.056 mole) and 6.3 Gm. (0.028 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.<sup>1</sup> The yellow colored product, distilling at 220–235°/0.1 mm., weighed 8.2 Gm. (80.6% yield);  $n_D^{25}$  1.5572.

A hydrochloride was prepared by adding, dropwise with stirring, an ethereal solution of hydrogen chloride to a solution of 1 Gm. of material dissolved in 25 ml. of absolute ether. The precipitated hydrochloride was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 165–166° (decompn.).

*Anal.*—Calcd. for  $C_{25}H_{50}NO_3 \cdot HCl$ : C, 69.07; H, 6.55. Found: C, 68.67; H, 6.49.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from an ethanol-acetone mixture. The picrate was dissolved in a minimum amount of acetone and an equal volume of commercial absolute ethanol was added to it. The solution was concentrated to one-half of its original volume and crystallization was allowed to take place. The recrystallized picrate was dried overnight at 100° under vacuum; m. p. 184–185° (decompn.).

*Anal.*—Calcd. for  $C_{25}H_{50}NO_3 \cdot C_6H_3N_3O_7$ : C, 58.78; H, 4.76. Found: C, 58.90; H, 4.96.

**Dicyclohexyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its Hydrochloride and Picrate.**—Anhydrous dicyclohexylmethanol (3.9 Gm., 0.02 mole) and 4.5 Gm. (0.02 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.<sup>1</sup> The yellow colored product, distilling at 185–200°/1 mm., weighed 5.2 Gm. (69.5% yield);  $n_D^{25}$  1.5001.

A hydrochloride was prepared by adding, dropwise with stirring, an ethereal solution of hydrogen chloride to a solution of 1 Gm. of material dissolved in 25 ml. of absolute ether. The precipitated hydrochloride was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 235–236° (decompn. at 190°).

*Anal.*—Calcd. for  $C_{25}H_{42}NO_3 \cdot HCl \cdot H_2O$ : C, 64.24; H, 9.31. Found: C, 64.57; H, 9.11.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated

<sup>2</sup> The residue was distilled directly without washing since a previous synthesis of the same compound indicated that it was too water-soluble to permit washing with water.

solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from absolute ethanol; m. p. 189-190°.

*Anal.*—Calcd. for  $C_{25}H_{12}NO_3 \cdot C_6H_5N_3O_7$ : C, 57.60; H, 6.67. Found: C, 57.90; H, 6.82.

**Cyclohexyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its *p*-Toluene Sulfonate and Picrate.**—Anhydrous cyclohexanol (5.6 Gm., 0.056 mole) and 6.3 Gm. (0.028 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C and the transesterification was accomplished by following the general procedure and using a modified Dean-Stark separator.<sup>1</sup> The yellow colored product, distilling at 165-175°/0.5 mm., weighed 5.4 Gm. (69.3% yield);  $n_D^{25}$  1.4961.

A *p*-toluene sulfonate was prepared by dissolving 1 Gm. of material in 25 ml. of absolute ether and adding, dropwise with stirring, a saturated solution of *p*-toluene sulfonic acid monohydrate in anhydrous ether. The precipitated *p*-toluene sulfonate was recrystallized four times from an acetone-isopropyl ether mixture and dried overnight at 100° under vacuum; m. p. 134-135°.

*Anal.*—Calcd. for  $C_{18}H_{30}NO_3 \cdot C_7H_5O_3S$ : C, 61.17; H, 7.37. Found: C, 61.10; H, 7.49.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding, dropwise with stirring, a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from commercial absolute ethanol and dried overnight at 100° under vacuum; m. p. 205-206° (decompn.).

*Anal.*—Calcd. for  $C_{18}H_{30}NO_3 \cdot C_6H_5N_3O_7$ : C, 51.96; H, 5.55. Found: C, 51.96; H, 5.55.

**1-Methyl-4-piperidyl 5,8-Methylimino-1-oxa-spiro-(2,6)-nonane-2-carboxylate and Its Methyl Bromide and Picrate.**—This compound was prepared by slightly modifying the general procedure for transesterification. Anhydrous 1-methyl-4-piperidinol (5.4 Gm., 0.047 mole) and 10.5 Gm. (0.047 mole) of II were dissolved in 150 ml. of anhydrous, redistilled Skellysolve C. After bringing the solution to reflux temperature and addition of the catalyst, a portion of distillate (350 ml.) was removed while simultaneously adding, dropwise, 350 ml. of fresh Skellysolve C. Reflux was continued, with stirring, for six hours, after which the Skellysolve C was removed by distillation under reduced pressure. The residue was distilled *in vacuo*.<sup>2</sup> The yellow colored product, distilling at 155-175°/0.45 mm., weighed 5.1 Gm. (36.9% yield);  $n_D^{25}$  1.4962.

A methyl bromide was prepared by dissolving 1 Gm. of material in 20 ml. of anhydrous ether and adding 20 ml. of methyl bromide. The mixture was kept three days at room temperature in a pressure bottle. The precipitated methyl bromide was filtered under dry nitrogen and recrystallized four times from a methanol-isopropyl ether mixture (filtering under dry nitrogen each time because of hygroscopicity). The very hygroscopic methyl bromide was dried overnight at 100° under vacuum. A melting point could not be obtained under 265°. The compound, however, decomposed with charring, over the range 230-265°.

*Anal.*—Calcd. for  $C_{18}H_{31}N_2O_3 \cdot 2CH_3Br \cdot 2H_2O$ : C, 41.16; H, 6.97. Found: C, 41.13; H, 7.19.

A picrate was prepared by dissolving 1 Gm. of material in 25 ml. of commercial absolute ethanol and adding it, dropwise with stirring, to 20 ml. of a saturated solution of picric acid in commercial absolute ethanol. The precipitated picrate was recrystallized four times from an acetone-ethanol mixture. The picrate first was dissolved in a minimum amount of acetone and an equal volume of commercial absolute ethanol was added to it. The resulting solution then was concentrated to one-half of its original volume and crystallization was allowed to take place. The recrystallized picrate was dried overnight at 100° under vacuum; m. p. 227-228° (decompn.).

*Anal.*—Calcd. for  $C_{18}H_{31}N_2O_3 \cdot 2C_6H_5N_3O_7$ : C, 44.68; H, 4.29. Found: C, 44.72; H, 4.32.

## PHARMACOLOGICAL EVALUATION

The apparatus for the pharmacological evaluation consisted of a glass cylinder 12 cm. high with an inside diameter of 5 cm. A large rubber stopper equipped with a glass drain tube was fitted into one end of the cylinder. Rubber tubing was attached to the glass drain tube and the whole was immersed in a constant temperature bath in such a manner that the cylinder could be drained of its contents by applying suction to the rubber tubing while the cylinder remained immersed in the bath. A metal rod with a muscle hook was placed inside the cylinder and a muscle lever was situated so that its hook was located directly above the hook inside the cylinder. The muscle lever was so arranged that it would trace on a revolving kymograph drum covered with smoked kymograph paper. A glass tube, connected to a supply of oxygen, was also placed inside the cylinder so that any solution inside the cylinder could be readily oxygenated. Locke's solution (150 ml.) was placed in the cylinder and the temperature bath was warmed to 38° with a thermostatically controlled water heater. The temperature was maintained at  $38 \pm 0.1^\circ$ . When the temperature of the Locke's solution had reached 38°, a flow of oxygen was started and a 4-cm. section of freshly excised rabbit ileum<sup>3</sup> was attached to the muscle hooks so that the muscle section was completely submerged in the warm Locke's solution. The kymograph drum was slowly rotated and 10 ml. of a 0.3% solution of Mecholyl chloride<sup>4</sup> was added to the Locke's solution. After the muscle had responded to the Mecholyl chloride, 0.1 ml. of a 1% solution of atropine sulfate was added. The same procedure was employed with the following, utilizing a fresh section of ileum and 150 ml. of fresh Locke's solution each time: 5 ml. of Mecholyl chloride (0.3%) followed by 0.2 ml. of compound P (1.2%), followed by an additional 0.2 ml. of compound P (1.2%); 10 ml. of Mecholyl chloride (0.3%) followed by 0.2 ml. of compound T (1.2%). All solutions were prepared by using recently boiled, distilled water as the solvent. The results of the pharmacological evaluation are shown in Fig. 1.

<sup>3</sup> The freshly excised rabbit ileum was stored in a cold solution of Locke's solution and rabbit blood.

<sup>4</sup> Trade name for methacholine chloride.

## SUMMARY

Eight new glycidic esters that may possess antispasmodic activity were prepared

Two new glycidic esters were pharmacologically screened for their antispasmodic activity with apparently positive results

Sodium borohydride was found to be effective in the reduction of 1-methyl-4-piperidone

Sodium hydride was found to be a convenient and efficient base in the Darzens condensation

Sodium hydride was found to be a good catalyst in the transesterification of the glycidic esters

## REFERENCES

- (1) Gyermek, L., and N'ador, K., *J Pharm and Pharmacol*, **9**, 209(1957)
- (2) Barlow, R. B., "Introduction to Chemical Phar-

- macology," John Wiley & Sons, Inc., New York, N. Y., 1955, p. 82
- (3) Lieberman, C., and Limpach, L., *Ber.*, **25**, 927(1892)
- (4) Stoll, A., and Jucker, E., *Angew Chem*, **66**, 376(1954)
- (5) Nachod, F. C., and Lands, A. M., *Trans N Y Acad Sci*, **16**, 2(1953)
- (6) Keagle, L. C., and Hartung, W. H., *J Am Chem Soc*, **68**, 1608(1946)
- (7) McElvain, S. M., and Rorig, K., *ibid*, **70**, 1820(1948)
- (8) Newman, M. S., in "Organic Reactions," Vol. 5, John Wiley & Sons, Inc., New York, N. Y., 1949, p. 414
- (9) Hunt, R. H., Chinn, L. J., and Johnson, W. S., *Org Syntheses*, **34**, 54(1954)
- (10) *Chem Eng News*, **36**, 125(1958)
- (11) Newman, M. S., in "Organic Reactions," Vol. 5, John Wiley & Sons, Inc., New York, N. Y., 1949, p. 427
- (12) Mousseron, M., and Granger, R., *Bull soc chim*, **1946**, 218
- (13) Sabatier, P., and Mailhe, A., *ibid*, **1905**, 74
- (14) Coleman, G. H., and Craig, D., *Org Syntheses*, **15**, 11(1935)
- (15) Bolyard, N. W., and McElvain, S. M., *J Am Chem Soc*, **51**, 922(1929)
- (16) Counsel, R. E., Ph. D. Thesis, University of Minnesota, 1957, p. 44
- (17) Counsel, R. E., *ibid*, University of Minnesota, 1957, p. 67
- (18) Toomey, R. F., and Riegel, R. E., *J Org Chem*, **17**, 1492(1952)

## A Quantitative Method for the Alkaloid of *Acacia berlandieri*\*

By BENNIE J. CAMP and JOAN A. MOORE

Using the reagent 1-fluoro-2,4-dinitrobenzene, a rapid and reliable method is described for the quantitative estimation of N-methyl beta-phenylethylamine in *Acacia berlandieri*. The method appears to be specific for the amine since paper chromatograms of plant extracts failed to indicate the presence of interfering amines and alkaloids.

**A**CACIA BERLANDIERI (guajillo) is a leguminous plant which contains a compound poisonous to certain animals. Camp and Lyman (1) isolated and identified the poisonous principle as N-methyl beta-phenylethylamine.

It became necessary to develop a quantitative method for the determination of this sympathomimetic compound before the following studies could be made: (a) the effects of environmental factors such as temperature and rainfall on the seasonal variation of the amine content of the plant, (b) the metabolism of the compound by microorganisms, (c) the deposition of the amine in certain mammalian tissues, and (d) the com-

plexing of the amine with organic and inorganic compounds.

McIntire, *et al* (2), demonstrated the use of 1-fluoro-2,4 dinitrobenzene as a quantitative reagent for certain primary and secondary amines. The purpose of this paper is to report a quantitative procedure for assaying the N-methyl beta-phenylethylamine content of *Acacia berlandieri*.

### EXPERIMENTAL

**Synthesis.**—Since this amine is not available commercially, it was necessary to synthesize the compound. To 5 L. of dry *n*-butyl alcohol containing 15 moles (465 Gm.) of anhydrous monomethylamine are added 3 moles (555 Gm.) of (2-bromomethyl) benzene. The vessel is stoppered and allowed to stand for twenty-four hours at room temperature. It is then heated on a steam bath to increase the rate of reaction. The monomethylamine hydrobromide which forms as a side product is removed by filtration, and the supernatant liquid is concentrated to approximately 25% of its original volume by *in vacuo* distillation. Upon cooling, the hydrobromide salt of the amine crystallizes from the *n*-butyl alcohol. The amine is converted to the hydrochloride salt by treating the *n*-butyl alcohol containing the amine hydrobromide salt with 40% sodium hydroxide until the alcohol phase becomes distinctly alkali-

\* Received August 21, 1959, from the Department of Biochemistry and Nutrition, Texas Agricultural Experiment Station, College Station.

This work was supported in part by a research grant from the Robert A. Welch Foundation, Houston, Tex. Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.



line. The free base formed by the addition of sodium hydroxide is readily soluble in the *n*-butyl alcohol phase, and the alcohol and aqueous phases are separated by means of a separatory funnel. The *n*-butyl alcohol is dried over anhydrous sodium sulfate, and anhydrous hydrogen chloride gas is passed into the alcohol solution until it becomes distinctly acid. If the *n*-butyl alcohol is not dry before the addition of the hydrogen chloride gas, then the hydrochloride salt will contain water of crystallization which melts at a lower temperature, 141–142°. The compound is purified by dissolving in chloroform and then filtered to remove small quantities of monomethylamine hydrochloride present. The hydrochloride salt crystallizes upon the addition of anhydrous ether to the chloroform solution. The compound is stored as the acid salt because the free base decomposes upon standing. The following melting points were obtained for derivatives prepared from the synthetic compound: hydrochloride, m.p. 157–158°; reported m. p. 157–158° (3), and 161–162° (4); picrate, m. p. 139–141°; reported m. p. 141–142° (4), and 141° (5).

The free base is formed by dissolving the acid salt in concentrated sodium hydroxide; the base forms an oily layer and is separated in a separatory funnel. It is washed several times with distilled water, dried over anhydrous sodium sulfate, and distilled under reduced pressure. It boils at 107–108° at 31 mm pressure; reported b. p. 67.4° at 3.25 mm. (2) and 112.5–115° at 36–40 mm. pressure (5).

**Standard Curve.**—The free amine is used to establish the standard curve. The procedure for preparing the 2,4-dinitrobenzene derivative is similar to the method described by McIntire, *et al* (2). A series of standards, ranging in amine content from 10 to 100  $\gamma$ , are prepared by transferring with a pipet the appropriate amount of the alcoholic solution of the amine to a series of 25-ml. volumetric flasks, each containing 0.10 ml. of 1% alcoholic FDNB (Eastman), 0.2 ml. of 0.2 *M* NaHCO<sub>3</sub>, 0.2 ml. absolute alcohol, and sufficient water to make a total volume of 1.5 ml. The flask is heated in a 60° water bath for twenty minutes. The excess reagent is then hydrolyzed by adding 0.5 ml. of 0.2 *N* NaOH and incubating an additional sixty minutes in the water bath. The contents of the flask are diluted to volume and extracted with 10 ml. of cyclohexane in a separatory funnel. The absorbance of the cyclohexane phase is read on a Beckman DU spectrophotometer at 350  $\mu$ , using a blank to adjust the instrument to zero absorbance. The blank, alcohol without amine, was developed in the same manner as the alcoholic amine standards. Figure 1 represents a typical standard curve. The relationship between the absorbance of the 2,4-dinitrobenzene derivative and the quantity of the amine used in the reaction is linear when the quantity of amine does not exceed 120  $\gamma$ . However, the relationship becomes curvilinear when the quantity of amine used in the reaction is greater than 120  $\gamma$ , not shown on curve.

**Assay of Plant Material.**—The air dried leaves from the acacia plant are ground in a Wiley mill to pass a 20-mesh screen. Five grams of the sample are extracted with 250 ml. of 1% HCl for approximately two hours in a 1-L. wide-mouth polyethylene bottle (3½ × 6½ inches) on a magnetic stirrer. The bottle and contents are centrifuged, and the

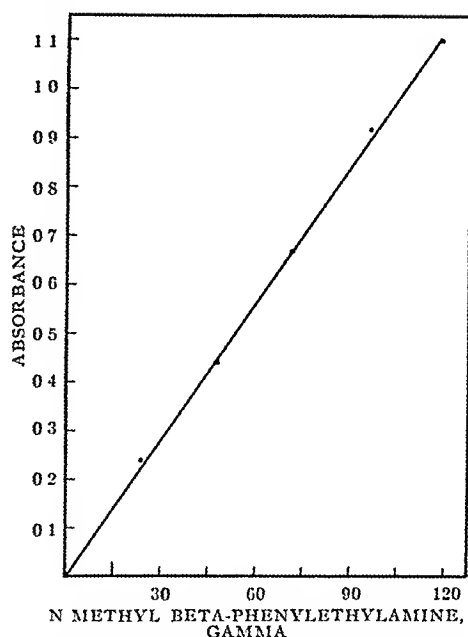


Fig. 1.—Relationship of absorbance of 2,4-dinitrobenzene derivative and quantity of amine used in reaction.

clear, supernatant liquid is decanted into a 500-ml. volumetric flask. It is re-extracted twice with 100 ml. of 1% HCl for one hour, centrifuged, and diluted to volume. A 100-ml. aliquot is adjusted to pH 10 with sodium hydroxide and extracted three times each with 100 ml. of chloroform. The combined chloroform extracts are made acid with 0.5 ml. of alcoholic HCl (alcohol saturated with hydrogen chloride gas) and reduced to dryness by vacuum distillation. The residue is dissolved in absolute alcohol and transferred quantitatively to a 25-ml. volumetric flask. It is neutralized with sodium hydroxide, and diluted to volume. A 0.2-ml. aliquot sample is used in the reaction with FDNB. The quantity of the amine in the aliquot sample is found by reading graphically from the standard curve the amount of amine equivalent to the absorbance of the FDNB derivative.

## RESULTS

Table I gives the per cent recovery when known quantities of the pure amine compound were carried through the chloroform extraction procedure and the converted to the 2,4-dinitrobenzene derivative.

TABLE I.—PER CENT RECOVERY OF AMINE COMPOUND

Sample	Theory, gamma/0.5 ml.	Found, <sup>a</sup> gamma/0.5 ml.	Theory, %
1	25	27.00	108.0
2	25	27.25	110.0
3	50	51.50	103.0
4	50	53.00	106.0

<sup>a</sup> Average of duplicate samples.



Table II presents the results of a recovery study when known quantities of the amine were added to plant extracts. An aliquot part of the sample was used which would fall within the 100  $\gamma$  range. Within this range, the average per cent recovery exceeds 95% and demonstrates the reliability of this method as a quantitative procedure for the amine content of this plant.

TABLE II—RECOVERY OF AMINE ADDED TO PLANT EXTRACT

Sample	Found Plant gamma/0.4 ml	Added gamma/0.4 ml	Total Found gamma	Re covered %
1	26.5	25.7	58.0 <sup>a</sup>	111.0
2	26.5	41.5	65.0	95.6
3	26.5	51.9	74.5	95.2
4	26.5	62.3	83.0	93.4

<sup>a</sup> Average of duplicate samples

To further test the reliability of this method, the month of July sample was repeatedly assayed, and the following values were obtained: 0.38, 0.44, 0.40, 0.40, 0.46, 0.42, 0.43, 0.46, 0.43, and 0.44%.

Leaf samples from the acacia plant were collected during the 1958 growing season to ascertain if any change occurred in the amine content of the plant during this period. Table III presents the results obtained from an analysis of leaf samples during 1958. A slight fluctuation occurs within the growing season, and samples are being collected for the 1959 growing season to determine if this fluctuation is correlated with environmental factors.

This method is currently being used in the author's laboratory to study the utilization of N-methyl-beta-phenylethylamine by microorganisms.

TABLE III—AMINE CONTENT OF LEAF SAMPLES FROM *Acacia berlandieri*

Sample	Amine % <sup>a</sup>
May	0.66
June	0.46
July	0.42
August	0.46
September	0.28
October	0.46

<sup>a</sup> Per cent based on weight of air dried sample

## SUMMARY

1. A method is described for a large scale synthesis of N-methyl-beta-phenylethylamine.

2. An analytical procedure is described for assaying the sympathomimetic amine content of *Acacia berlandieri*.

3. Data are given showing the amine content of leaf samples during the 1958 growing season.

## REFERENCES

- (1) Camp B. J. and Lyman C. M. *THIS JOURNAL* 45, 719 (1956).
- (2) McIntire F. C., Clements L. M. and Sproull M. *Anal. Chem.* 25, 1757 (1953).
- (3) Durkee H. and Becker P. *Ann. Chem. Liebigs* 395, 368 (1912).
- (4) Yuraskevskii N. K. *J. Gen. Chem. (U. S. S. R.)* 11, 157 (1941); *Chem. Abstr.* 35, 5503 (1941).
- (5) Johnson T. B. and Guest H. G. *Am. Chem. J.* 42, 345 (1909).

# A Simplified Colorimetric Method for Determination of Mercury in Biologic Materials\*

By DONALD L. SORBY and ELMER M. PLEIN

A simplified colorimetric method for determination of mercury by the dithizone method has been developed. The procedure is suitable for analyzing biological and certain mineral samples for their mercury content. Amounts of mercury between 10 and 100 mcg. have been analyzed with a maximum deviation of  $\pm 5$  per cent. The procedure has been simplified wherever possible to do so without sacrificing accuracy.

FOR AN INVESTIGATION of the penetration of ammoniated mercury through the skin of laboratory animals, a simple and rapid procedure for determining mercury in biologic material was desired. Various procedures were investigated (1-6) and were found to be rather time

consuming or not of the desired selectivity. The method presented here combines the most desirable features of two of these methods (3, 4).

The digestion procedure of Polley and Miller (4) was chosen because of its simplicity and relatively mild conditions. For determination of mercury in the digest solutions the procedure of Plein and Plein (3) was utilized because this method is quite simple and requires comparatively little time to carry out.

## EXPERIMENTAL

**Reagents.**—Chloroform is distilled in glass apparatus and 0.75% ethanol is added as a preservative, sulfuric acid, concentrated and 0.5 N, hydro

\* Received August 17, 1959, from the University of Washington, College of Pharmacy, Seattle.

gen peroxide, 90%, solutions of approximately 50% strength are freshly prepared by dilution with distilled water; dithizone solution, diphenylthiocarbazone (Eastman white label), is dissolved (1 mg. per ml.) in chloroform and refrigerated, fresh dilutions of this solution containing 4 mg. per liter are prepared for each assay; hydrochloric acid, 0.25 *N*; hydroxylamine hydrochloride solution, 20%, purified by shaking with several portions of a chloroform solution of dithizone (10 mg. per ml.) and washing with chloroform until the green color is removed; potassium permanganate, 5%; standard mercuric chloride solution, 1,000 mcg. mercury per ml. mercuric chloride (0.1354 Gm.) is dissolved in 100 ml. of 0.5 *N* sulfuric acid, and this solution is diluted 1:100 for use in preparing a standard curve for each determination.

Laug, *et al.* (1), recommended the use of glass distilled water in order to avoid interference of copper in the assay. Water for this experiment was obtained from a Barnstead type "Q" still. Water from this source was found to be satisfactory for use in the dithizone procedure.

**Digestion of the Sample.**—The digestion procedure differs somewhat for each type of sample. Such factors as moisture content, amount of organic matter present, physical form of the sample, and presence of undigestible material require slight modifications of the following procedure to fit the particular sample.

A one-gram sample is placed in a 250-ml. flat-bottomed standard-taper flask and an Ahlin condenser is fitted to the flask. Five milliliters concentrated sulfuric acid is added through the condenser. The apparatus is heated on a steam bath and 50% hydrogen peroxide is added, dropwise, through the condenser with swirling of the sample. The dropwise addition of the hydrogen peroxide is continued until the solution becomes clear and nearly colorless. The steam bath is then removed and the flask is heated on an asbestos pad over a low flame. Drops of 50% hydrogen peroxide are added and heating is continued as the solution darkens. Heating is discontinued when the samples remain clear and colorless for two to three minutes after the last addition of hydrogen peroxide. Ten milliliters of water is added through the condenser after the solution has cooled. The flask is removed from the condenser and 5% potassium permanganate is added, dropwise, with swirling until a pink color persists. Ten milliliters of 20% hydroxylamine hydrochloride solution is then added to the flask. The sample is filtered into a 50-ml. volumetric flask and the digestion flask is rinsed with several portions of water. The rinsings are added to the solution in the volumetric flask and the samples are made to volume with distilled water.

**Determination.**—Fifty milliliters of 0.25 *N* hydrochloric acid is placed in a 250-ml. separatory funnel. The proper aliquot of the digested solution is added and the contents of the funnel are mixed well. Twenty-five milliliters of dithizone solution (4 mg. per liter) is added and the contents of the funnel are shaken 60 times. A pledget of absorbent cotton is inserted into the outlet of the separatory funnel. The colored chloroform layer is then strained through the cotton into a sample cuvet and the

absorbance of the solution is determined at 490  $m\mu$  with the Beckman DU spectrophotometer.

Aliquots are usually chosen so that the sample for determination will contain 10 to 25 mcg. mercury.

The amount of mercury in the digested solution is determined by comparison with a standard curve prepared according to the following procedure. Fifty milliliters of 0.25 *N* hydrochloric acid is placed in a 250-ml. separatory funnel and concentrated sulfuric acid equivalent to the amount of acid theoretically present in the aliquot taken is added. One milliliter of 20% hydroxylamine hydrochloride is added for each ten ml. of aliquot used. Finally, the proper amount of standard mercury solution is added and the solutions are mixed well. Twenty-five milliliters of dithizone solution is added and the absorbance is determined as described above. The standard curve follows Beer's law up to a concentration of 30 mcg. in the aliquot. For larger mercury concentrations a smaller aliquot must be chosen. If a large aliquot is used, enough water should be added to the standard preparations so that their volume will be approximately equal to the digest samples.

## DISCUSSION

When samples are low in moisture content and high in organic matter a 10-ml. quantity of concentrated sulfuric acid is usually necessary. If the samples are observed to be digesting slowly the second 5-ml. portion of acid is added through the condenser. Polley and Miller (4) list a number of different types of material and the variations in conditions of the digestion procedure necessary for each.

The size of the sample chosen may be varied according to mercury content. If larger samples are used the amount of acid must be increased.

Additions of hydrogen peroxide must be made very carefully to prevent a vigorous reaction. Expulsion of gas from the condenser is to be avoided. Great care is also necessary when heating the samples over the open flame, and should be only vigorous enough to cause the reaction to proceed. Excessively high and prolonged heating was found to reduce greatly the amount of mercury recovered from the samples.

The nature of the samples determines the filtration treatment necessary for the digests. Samples which are high in fat content may be chilled prior to filtration to solidify the fats. Samples with no insoluble residue need not be filtered. Filtrations are best accomplished through a fine-grade sintered-glass filter utilizing reduced pressure.

Polley and Miller recommended addition of sodium chloride to the samples after digestion and during the determination procedure to prevent adsorption of mercury onto the glass walls of containers and onto silica of soil samples. Under conditions of this experiment no adsorption of mercury could be detected, even though the samples were allowed to remain in the separatory funnels for periods up to thirty-six hours. Thus, further addition of chloride ion to the samples was unnecessary.

The color of the mercury-dithizone complex was stable up to one and one-half hours wh

TABLE I.—RECOVERY OF KNOWN AMOUNTS OF MERCURY FROM SAMPLES (MERCURY ADDED AS MERCURIC CHLORIDE)

Material	No. of Samples	Mercury Added, mcg.	Mercury Found, mcg. (Mean)	Max. Deviation, mcg.
Beef muscle	7 <sup>a</sup>	100	98.7 ± 2.0 <sup>b</sup>	-5.0
Beef muscle	7	10 <sup>c</sup>	9.7 ± 0.2	-0.5
			9.7 ± 0.1	-0.4
Rhubarb root	7	100	101.4 ± 0.9	+3.0
Soil	7 <sup>d</sup>	100	99.9 ± 0.8	+1.0

<sup>a</sup> For each material, an eighth sample containing no added mercury was carried through the procedure. Only in the case of the one-fifth aliquot of the 10 mcg beef muscle sample was mercury (1.0 mcg.) found in these samples.

<sup>b</sup> The standard deviation of the mean was calculated by the formula  $\sigma = \sqrt{\frac{1}{n-1} \sum d^2}$ .

<sup>c</sup> The first value was obtained using a one-fifth aliquot of the sample digest. The second value was obtained using a one-half aliquot.

<sup>d</sup> The samples were prepared with soil collected in one area.

TABLE II.—RECOVERY OF KNOWN AMOUNTS OF ORGANIC MERCURIAL

Mercurial	No of Samples	Mercury Added, mcg.	Mercury Found, mcg. (Mean)	Max. Deviation, mcg.
Di- <i>p</i> -tolylmercury	4	100	100.8 ± 0.8	+1.5
Sodium ethylmercurithiosalicylate	4	100	100.6 ± 1.3	+2.5

stored in normal lighting conditions of the laboratory. No study was made on the effect of strong sunlight or artificial light. Strong light is probably best avoided however.

The effect of various metals which might possibly interfere in the determination of mercury was investigated. One milligram quantities of iron, nickel, lead, copper, zinc, cadmium, manganese, and bismuth were found to have no effect on the assay.

Although the procedure was developed primarily for use with animal tissue, the applicability of the method to certain mineral and vegetable materials was also tested. The results are summarized in Table I.

The procedure was tested to determine if it would be sensitive to organic mercurials as well as inorganic mercury. The results are summarized in Table II.

The accuracy of the experiment using the Beckman DU spectrophotometer seems to be com-

parable to that reported by Polley and Miller (4) i. e., 1 mcg. or 5%, whichever is greater. The overall time required for the whole procedure is approximately five hours depending upon the type of sample, the number of samples, and the experience of the operator. Since the procedure is less complicated and requires less manipulation of the samples than many other methods, there is less chance for loss of small amounts of sample and the consequent errors involved.

REFERENCES

(1) Laug, E. P., and Nelson, K. W., *J. Assoc. Offic. Agr. Chemists*, 25, 399(1942).  
(2) Maren, T. H., *J. Lab. Clin. Med.*, 28, 511(1953).  
(3) Plein, J. B., and Plein, E. M., *THIS JOURNAL*, 46, 705(1957).  
(4) Polley, D., and Miller, V. L., *Anal. Chem.*, 27, 1162(1955).  
(5) Simonsen, D. G., *Am. J. Clin. Pathol.*, 23, 789(1953).  
(6) Winkler, W. O., *J. Assoc. Offic. Agr. Chemists*, 21, 220(1938).

# Color Stability of Tablet Formulations I\*

## Measurement of Changes in Individual Tablet Reflectance

By TIBOR URBANYI, CHARLES J. SWARTZ, and LEON LACHMAN

A holder which permits the centering and leveling of individual tablets in the reflectance attachment of the Beckman model DU spectrophotometer is described. By this adaptation, it is possible to measure the reflected light from the upper or lower surface of a tablet with a precision of two per cent. This method of measurement has proved useful in assigning reproducible numerical values for the light reflected from tablets.

A PROMINENT CONSIDERATION in the stability evaluation of compressed tablets is the establishment of a tablet formulation which exhibits minimum alterations in color with storage. Although this may be desirable, it is a recognized fact that many tablet dosage forms showed change in color with time. Despite this, there are apparently no reports in the literature concerning the measurement of changes in appearance of tablets. An important reason for this situation may be the unavailability of suitable analytical techniques for measurement of color changes in individual tablets. It was, therefore, the purpose of this investigation to develop an analytical technique to accomplish the measurement of the aforementioned property.

Methods available for the standardization and evaluation of the appearance of tablets can be classified into two categories, visual and instrumental. Visual estimation of tablet color is accomplished by comparison with standard tablets of the desired color or comparison with artificial standards such as color chips or charts. Comparison with standard tablets is unsatisfactory due to the possibility of subtle changes in the standard with time, while with color chips or standard reference charts of color, it is almost impossible to obtain these items to match the tablets under study.

Although the human eye is superior to instruments in measuring small hue and chroma differences, spectrophotometers are capable of more precise detection of small value differences (1). In order to study and predict shelf life of tablets from an appearance standpoint, an instrumental method capable of defining color intensity in reliable, reproducible values was indicated. The presently used instruments such as the "Color Eye" require relatively large samples which may be advantageous for batch control. However, because of the variations that may

exist in color intensity between faces of single tablets and the resulting void spaces that form when several tablets are placed in the holder at one time, a method for individual tablets was desired. The method should define the appearance of the tablet in precise reproducible terms and be applicable to either the upper or lower face of the tablet. For this purpose a procedure was developed and a description, as well as representative data, obtained through the use of this method are presented.

### EXPERIMENTAL

**Description of Tablet Holder.**—Since practically all analytical laboratories are equipped with highly accurate and precise spectrophotometers which can be used for the determinations of the reflectance of powders, it was decided to utilize the Beckman model DU spectrophotometer and its reflectance attachment for the measurement of the reflectance of individual tablets. In order to accomplish this, a tablet holder was designed to permit accurate, reproducible centering and leveling of the individual tablet in the reflectance attachment. Such a holder is shown in Fig. 1. The holder is made of oxidized steel with dimension *A* being the diameter of the tablet.

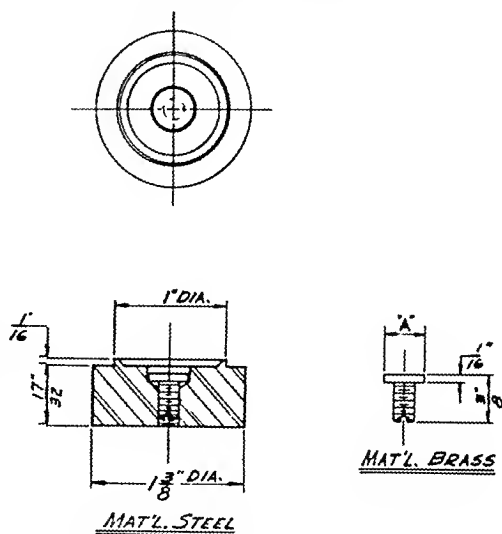


Fig. 1.—Tablet holder for reflectance attachment.

\* Received August 21, 1959, from the Research Department, CIBA Pharmaceutical Products, Inc., Summit, N. J. Presented to the Scientific Section, A. Ph. A. Cincinnati meeting, August 1959.

With minor modifications the holder can be adapted to other spectrophotometers

**Procedure for Measurement.**—The tablet to be measured is placed in the tablet holder and the leveling screw of the holder is adjusted so that the top edge of the tablet is made level with the holder. The holder is mounted in the front compartment of the reflectance attachment, and a National Bureau of Standards Vitrolite plate of known reflectance is placed in the back compartment. The diffuse reflectance is measured in accordance with standard techniques. The reflectance can be measured in absorbance or per cent transmission at the desired wavelength.

RESULTS AND DISCUSSION

A single tablet colored with FD&C Blue No. 1 was measured at its absorption maximum, 640 mμ, for a total of ten times, repositioning the tablet for each measurement of the upper and lower surface. The mean absorbance of the upper surface was 0.428 and the average deviation ±0.001, while the value for the lower face was 0.438 and the average deviation ±0.001 as represented in Table I. In a subsequent experiment ten different tablets from a single batch colored with FD&C Violet No. 1 were measured at their absorption maximum of 590 mμ. The mean absorbance at the upper face was 0.410 and the average deviation ±0.002, whereas the value for the lower face was 0.420 and the average deviation ±0.002 as shown in Table II. It is evident from the results presented in Tables I and II that excellent precision is obtained in the utilization of this technique for individual tablet measurement.

The diffuse reflectance can be measured at any

suitable wavelength. In the case of tablets colored with a single dye, measurement at the absorption maximum of the dye is usually satisfactory. The variation of the reflectance of FD&C Violet No. 1 with wavelength is illustrated in Fig. 2.

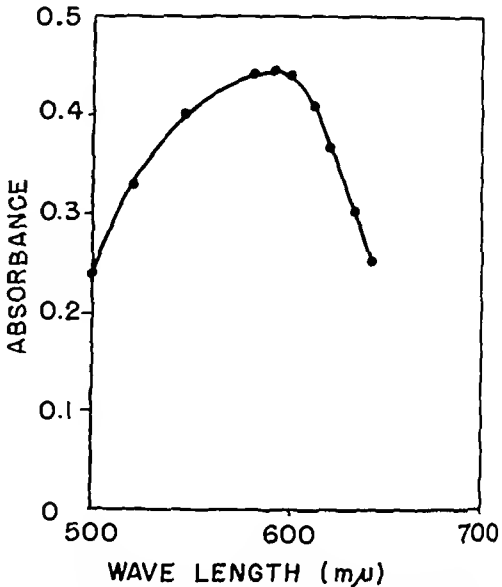


Fig. 2—A plot showing the variation of absorbance with wavelength for tablets colored with FD&C Violet No. 1

TABLE I—ABSORBANCE AT 640 mμ OF SINGLE TABLET COLORED WITH FD&C BLUE NO. 1

Absorbance Upper Face	Absorbance Lower Face
0.427	0.437
0.427	0.437
0.428	0.438
0.428	0.438
0.428	0.438
0.428	0.438
0.428	0.438
0.428	0.439
0.429	0.439
0.430	0.439

TABLE II—ABSORBANCE AT 590 mμ OF TEN TABLETS COLORED WITH FD&C VIOLET NO. 1

Tablet	Absorbance Upper Face	Absorbance Lower Face
1	0.408	0.417
2	0.408	0.418
3	0.409	0.419
4	0.409	0.420
5	0.410	0.420
6	0.410	0.420
7	0.411	0.421
8	0.411	0.421
9	0.411	0.421
10	0.412	0.422

TABLE III—TRISTIMULUS POINTS ON FIVE COLORED TABLETS

Wave length, mμ	FD&C Violet No. 1	FD&C Green No. 3	FD&C Blue No. 1	FD&C Red No. 1	D&C Yellow No. 10
640	0.226	0.436	0.432	0.036	0.046
546	0.367	0.200	0.166	0.230	0.055
436	0.091	0.196	0.100	0.161	0.260

For tablets containing more than one dye or an excipient which may fade or darken, it may be preferable to measure at the tristimulus points (normally 436, 546, and 640 mμ) or in the yellow, blue, and red regions.

Table III lists the absorbances obtained at the tristimulus points for five different colored tablets. When utilizing the tristimulus technique, it is desirable to plot the data on triangular graph paper in order to obtain a better visualization of the changes in hue that result.

This procedure has been found useful in the determination of the color stability of tablets containing several different certified dyes and is reported in a subsequent paper of this series (2).

REFERENCES

(1) Nickerson, D., Symposium On Color Difference Specifications, presented at a meeting of Committee E12 on Appearance, American Society for Testing Materials, Cleveland, Ohio, March 5-6, 1952.  
(2) Lachman, L., Swartz, C. J., Urbanyi, T., and Cooper, J. THIS JOURNAL, 49, 165(1960).

# Color Stability of Tablet Formulations II\*

## Influence of Light Intensity on the Fading of Several Water-Soluble Dyes

By LEON LACHMAN, CHARLES J. SWARTZ, TIBOR URBANYI, and JACK COOPER

The photosensitivity of a number of commonly used, certified water-soluble dyes employed in tablets has been investigated. Exposures were made under both normal and exaggerated light intensities. In both cases the spectral character of the radiation was similar. The dyes evaluated included FD&C Red No. 1, FD&C Red No. 3, D&C Green No. 5, FD&C Green No. 3, FD&C Blue No. 2, FD&C Blue No. 1, FD&C Yellow No. 5, D&C Yellow No. 10, D&C Orange No. 3, and FD&C Violet No. 1. Apparent rates of fading for the several dyes were determined. The relationship between fading and dye structure as well as fading and light intensity is discussed. The extent of dye fading beneath the surface of the tablet was also measured.

THE USE OF certified water-soluble dyes for imparting color to tablets is widespread in the pharmaceutical industry. The photosensitivity of these dyes, when incorporated into tablet formulations, represents a formidable problem. Although this is a generally recognized difficulty, there exists a conspicuous lack of information in the pharmaceutical literature concerning the influence of light on color fastness. This lack of data can be ascribed to several factors among which are (a) the unavailability of a suitable exaggerated-light stability cabinet, (b) the unavailability of appropriate analytical techniques for measurement of color changes at the surface of tablets, (c) the common practice of discontinuing the use of a specific color when found unsuitable in a given formulation without determining the nature of the objectionable characteristic, and (d) the treatment of such data as confidential within the particular company.

Because of the practical significance of this problem, a vast store of empirical knowledge on the subject has been accumulated in the pharmaceutical as well as other industries. Although pharmaceutical references are sparse, numerous reports exist on the nature of fading reactions in the textile, dye, and plastic industries. In summarizing this information certain aspects predominate. Depending upon their chemical constitution, dyes behave in many different ways under the influence of light. They may fluoresce (1), be oxidized or reduced, either reversibly or irreversibly (2, 3), or may sensitize the reaction of other components of the irradiated system (4, 5). Furthermore, the quality and intensity of light as well as the continuous or

intermittent nature of exposure strongly affect the fading reaction (6).

In light of recent Food and Drug Administration rulings regarding the decertification of many commonly used colorants, maximum utilization of all approved dyes now seems imperative. In order to accomplish this it is essential to ascertain the stability characteristics of the remaining certified dyes.

A study was therefore initiated to determine the light stability of ten certified water-soluble colors when used in tablet dosage forms. The purpose of this study was not the elucidation of specific fading mechanisms of the several colors but instead the determination of the relative stability of the colors under normal and exaggerated illumination. The basic tablet formulation used for this investigation was composed of relatively inert materials. The fading at the surface of the tablet was determined following exposure to so-called "normal" and "exaggerated" light intensities. Apparent rates of fading for the several colored tablets following exposure were calculated. The relationship of degree of fading at various depths from the tablet surface was measured for representative tablets. The possibility of the existence of a general relationship between the extent of fading under ordinary and accelerated illumination is discussed.

### EXPERIMENTAL

**Materials Used.**—FD&C Red No. 1, FD&C Red No. 3, D&C Green No. 5, FD&C Green No. 3, FD&C Blue No. 2, FD&C Blue No. 1, FD&C Yellow No. 5, D&C Yellow No. 10, D&C Orange No. 3, and FD&C Violet No. 1.

**Equipment.**—Light stability cabinets exhibiting intensified and normal room illumination as described in a previous publication (7), Gossen Tri Lux foot candle light meter, Stokes model F tablet

\* Received August 21, 1959, from the Research Department, CIBA Pharmaceutical Products, Inc., Summit, N. J. Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

With minor modifications the holder can be adapted to other spectrophotometers

**Procedure for Measurement.**—The tablet to be measured is placed in the tablet holder and the leveling screw of the holder is adjusted so that the top edge of the tablet is made level with the holder. The holder is mounted in the front compartment of the reflectance attachment, and a National Bureau of Standards Vitrolite plate of known reflectance is placed in the back compartment. The diffuse reflectance is measured in accordance with standard techniques. The reflectance can be measured in absorbance or per cent transmission at the desired wavelength.

## RESULTS AND DISCUSSION

A single tablet colored with FD&C Blue No 1 was measured at its absorption maximum, 640  $m\mu$ , for a total of ten times, repositioning the tablet for each measurement of the upper and lower surface. The mean absorbance of the upper surface was 0.428 and the average deviation  $\pm 0.001$ , while the value for the lower face was 0.438 and the average deviation  $\pm 0.001$  as represented in Table I. In a subsequent experiment ten different tablets from a single batch colored with FD&C Violet No 1 were measured at their absorption maximum of 590  $m\mu$ . The mean absorbance at the upper face was 0.410 and the average deviation  $\pm 0.002$ , whereas the value for the lower face was 0.420 and the average deviation  $\pm 0.002$  as shown in Table II. It is evident from the results presented in Tables I and II that excellent precision is obtained in the utilization of this technique for individual tablet measurement.

The diffuse reflectance can be measured at any

suitable wavelength. In the case of tablets colored with a single dye, measurement at the absorption maximum of the dye is usually satisfactory. The variation of the reflectance of FD&C Violet No 1 with wavelength is illustrated in Fig. 2.

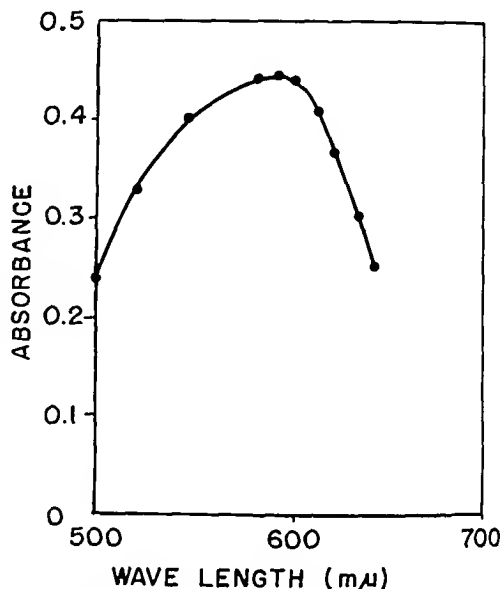


Fig 2—A plot showing the variation of absorbance with wavelength for tablets colored with FD&C Violet No 1

TABLE I.—ABSORBANCE AT 640  $m\mu$  OF SINGLE TABLET COLORED WITH FD&C BLUE NO 1

Absorbance Upper Face	Absorbance Lower Face
0.427	0.437
0.427	0.437
0.428	0.438
0.428	0.438
0.428	0.438
0.428	0.438
0.428	0.438
0.428	0.438
0.428	0.439
0.429	0.439
0.430	0.439

TABLE II.—ABSORBANCE AT 590  $m\mu$  OF TEN TABLETS COLORED WITH FD&C VIOLET NO 1

Tablet	Absorbance Upper Face	Absorbance Lower Face
1	0.408	0.417
2	0.408	0.418
3	0.409	0.419
4	0.409	0.420
5	0.410	0.420
6	0.410	0.420
7	0.411	0.421
8	0.411	0.421
9	0.411	0.421
10	0.412	0.422

TABLE III.—TRISTIMULUS POINTS ON FIVE COLORED TABLETS

Wave length, $m\mu$	FD&C Violet No 1	FD&C Green No 3	FD&C Blue No 1	FD&C Red No 1	D&C Yellow No 10
640	0.226	0.436	0.432	0.036	0.046
546	0.367	0.200	0.166	0.230	0.055
436	0.091	0.196	0.100	0.161	0.260

For tablets containing more than one dye or an excipient which may fade or darken, it may be preferable to measure at the tristimulus points (normally 436, 546, and 640  $m\mu$ ) or in the yellow, blue, and red regions.

Table III lists the absorbances obtained at the tristimulus points for five different colored tablets. When utilizing the tristimulus technique, it is desirable to plot the data on triangular graph paper in order to obtain a better visualization of the changes in hue that result.

This procedure has been found useful in the determination of the color stability of tablets containing several different certified dyes and is reported in a subsequent paper of this series (2).

## REFERENCES

- (1) Nickerson, D., Symposium On Color Difference Specifications, presented at a meeting of Committee E12 on Appearance, American Society for Testing Materials, Cleveland, Ohio, March 5-6, 1952.
- (2) Lachman, L., Swartz, C. J., Urbanyi, T., and Cooper, J. THIS JOURNAL, 49, 165 (1960)

# Color Stability of Tablet Formulations II\*

## Influence of Light Intensity on the Fading of Several Water-Soluble Dyes

LEON LACHMAN, CHARLES J. SWARTZ, TIBOR URBANYI, and JACK COOPER

The photosensitivity of a number of commonly used, certified water-soluble dyes employed in tablets has been investigated. Exposures were made under both normal and exaggerated light intensities. In both cases the spectral character of the radiation was similar. The dyes evaluated included FD&C Red No. 1, FD&C Red No. 3, D&C Green No. 5, FD&C Green No. 3, FD&C Blue No. 2, FD&C Blue No. 1, FD&C Yellow No. 5, D&C Yellow No. 10, D&C Orange No. 3, and FD&C Violet No. 1. Apparent rates of fading for the several dyes were determined. The relationship between fading and dye structure as well as fading and light intensity is discussed. The extent of dye fading beneath the surface of the tablet was also measured.

THE USE OF certified water-soluble dyes for imparting color to tablets is widespread in the pharmaceutical industry. The photosensitivity of these dyes, when incorporated into tablet formulations, represents a formidable problem. Although this is a generally recognized difficulty, there exists a conspicuous lack of information in the pharmaceutical literature concerning the influence of light on color fastness. This lack of data can be ascribed to several factors among which are (a) the unavailability of a suitable exaggerated-light stability cabinet, (b) the unavailability of appropriate analytical techniques for measurement of color changes at the surface of tablets, (c) the common practice of discontinuing the use of a specific color when found unsuitable in a given formulation without determining the nature of the objectionable characteristic, and (d) the treatment of such data as confidential within the particular company.

Because of the practical significance of this problem, a vast store of empirical knowledge on the subject has been accumulated in the pharmaceutical as well as other industries. Although pharmaceutical references are sparse, numerous reports exist on the nature of fading reactions in the textile, dye, and plastic industries. In summarizing this information certain aspects predominate. Depending upon their chemical constitution, dyes behave in many different ways under the influence of light. They may discolor (1), be oxidized or reduced, either reversibly or irreversibly (2, 3), or may sensitize the reaction of other components of the irradiated system (4, 5). Furthermore, the quality and intensity of light as well as the continuous or

intermittent nature of exposure strongly affect the fading reaction (6).

In light of recent Food and Drug Administration rulings regarding the decertification of many commonly used colorants, maximum utilization of all approved dyes now seems imperative. In order to accomplish this it is essential to ascertain the stability characteristics of the remaining certified dyes.

A study was therefore initiated to determine the light stability of ten certified water-soluble colors when used in tablet dosage forms. The purpose of this study was not the elucidation of specific fading mechanisms of the several colors but instead the determination of the relative stability of the colors under normal and exaggerated illumination. The basic tablet formulation used for this investigation was composed of relatively inert materials. The fading at the surface of the tablet was determined following exposure to so-called "normal" and "exaggerated" light intensities. Apparent rates of fading for the several colored tablets following exposure were calculated. The relationship of degree of fading at various depths from the tablet surface was measured for representative tablets. The possibility of the existence of a general relationship between the extent of fading under ordinary and accelerated illumination is discussed.

### EXPERIMENTAL

**Materials Used.**—FD&C Red No. 1, FD&C Red No. 3, D&C Green No. 5, FD&C Green No. 3, FD&C Blue No. 2, FD&C Blue No. 1, FD&C Yellow No. 5, D&C Yellow No. 10, D&C Orange No. 3, and FD&C Violet No. 1.

**Equipment.**—Light stability cabinets exhibiting intensified and normal room illumination as described in a previous publication (7), Gossen Tri Lux foot candle light meter, Stokes model F tablet

\* Received August 21, 1959, from the Research Department, CIBA Pharmaceutical Products, Inc., Summit, N. J. Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.



press, Beckman spectrophotometer model DU with reflectance attachment, die attachment for centering and leveling tablets in reflectance units as described in previous paper of this series (8), precision rotary microtome, and Beckman aquameter.

**Preparation of Tablets.**—Tablets containing the various water-soluble certified dyes were prepared according to the following formula:

Calcium sulfate, dihydrate. . . . .	96 99%
Tragacanth U. S. P. . . . .	2.00%
Magnesium stearate, U. S. P. . . . .	1 00%
Dye. . . . .	0 01%
Ethyl alcohol 50%. . . . .	q. s.

A granulation of the above formula was prepared in accordance with commonly-employed tableting procedures. The dye was dispersed by dissolving it in the 50% alcohol which was used as a granulating agent. Tablets weighing 700 mg. were compressed using  $16/32$  inch flat punches, uppers bisected. The tablets were all compressed to a hardness of approximately 7 Kg/in.<sup>2</sup> and a thickness of 3 mm. on a Stokes model F tablet press. The moisture content of the dried granulations was kept consistently below 0.5%.

**Storage of Samples in the Light Stability Cabinets.**—Adequate samples of the several differently colored tablets were put into three-inch uncovered clear-glass Petri dishes which were then placed into the normal and intensified light cabinets. The intensity of the light incident on the tablets in the respective cabinets was kept at a constant level through measurement of the light intensity with a Gossen Tri Lux meter at regular time intervals. The light intensities falling on the tablets in the normal and exaggerated illumination cabinets were 45 and 550 foot candles, respectively. Tablet samples were withdrawn from the cabinets at designated time intervals for reflectance measurements.

**Measurement of Tablet Reflectance.**—The tablets removed from the light stability cabinets were measured for fading of the color at the tablet surface by the use of a Beckman model DU spectrophotometer with a modified Beckman reflectance unit attachment to permit its use for single tablets. The equipment modifications and method of measurement are described in detail in a previous publication (8). Reflectance measurements were made at 640  $\mu$  for FD&C Blue No. 1, at 500  $\mu$  for FD&C Red No. 1, at 540  $\mu$  for FD&C Red No. 3, at 620  $\mu$  for FD&C Green No. 3, at 630  $\mu$  for D&C Green No. 5, at 420  $\mu$  for D&C Yellow No. 1, at 430  $\mu$  for FD&C Yellow No. 5, at 610  $\mu$  for FD&C Blue No. 2, at 590  $\mu$  for FD&C Violet No. 1, and at 500  $\mu$  for D&C Orange No. 3.

**Extent of Fading Beneath Surface of Tablet.**—In order to determine the degree of fading at various distances from the surface of the tablet individual tablets were sliced to definite thicknesses with a microtome. Each tablet was placed in a die which was subsequently leveled in the microtome to permit uniform vertical cuts of the flat surface of the tablet. Reflectance measurements were performed for the several colored tablets at distances up to 300  $\mu$  from the surface.

**Determination of Moisture Content of Tablets.**—Water contained in the tablets was measured by Karl Fischer titration, the end point being determined

electrometrically utilizing the Beckman aquameter. Sample tablets were crushed in a mortar and weighed aliquots removed for dispersion in methanol. This dispersion, under constant stirring, was then titrated with Karl Fischer reagent.

## RESULTS AND DISCUSSION

The photosensitivity of the several dyes used in the tablets studied was determined by exposure to normal and intensified illumination.

Representative plots showing a decrease in the spectral absorbance of the surface of the tablets colored with FD&C Blue No. 1, FD&C Yellow No. 5 and FD&C Blue No. 2 are presented in Figs. 1, 2 and 3. It can be seen from these curves that a substantial variance in the degree of color fading exists depending upon whether the tablets are exposed under normal or exaggerated illumination. In addition, the rate of fading of the surface color of the tablets is not the same for the several dyes studied, but varies according to the individual color used. An interesting fact brought out by these curves is the change that results in the absorption maximum as the fading progresses. A flattening as well as a hypsochromic shift of the absorption maximum occurs. The magnitude of this effect is greatest for FD&C Blue No. 1 and least for FD&C Blue No. 2, with FD&C Yellow No. 5 falling in between.

Representative plots indicating the rate of fading of dye from the surface of the tablets are given in Figs. 4 and 5. It is readily evident from the linearity of the plots that the fading follows an apparent first-order reaction. These graphs further show the presence of more than one slope for the plots of log absorbance vs. time for most of the samples studied. In all cases, the initial rate of fading was greater than that obtained from subsequent portions of the fading curve. Baxter, *et al.* (9), in their studies of the fastness of dyes in textiles also found fading to

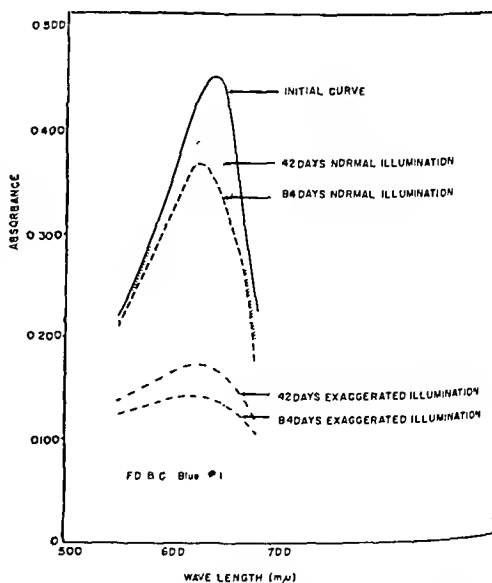


Fig. 1.—Plots of the visible absorption spectra of FD&C Blue No. 1 after different intervals of storage under normal and exaggerated illumination.

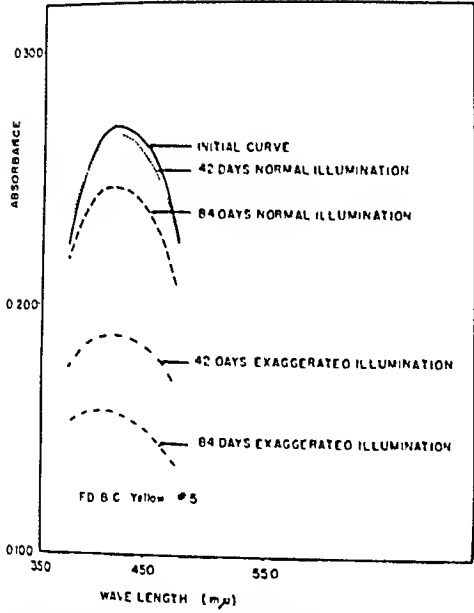


Fig 2—Plots of the visible absorption spectra of FD&C Yellow No. 5 after different intervals of storage under normal and exaggerated illumination.

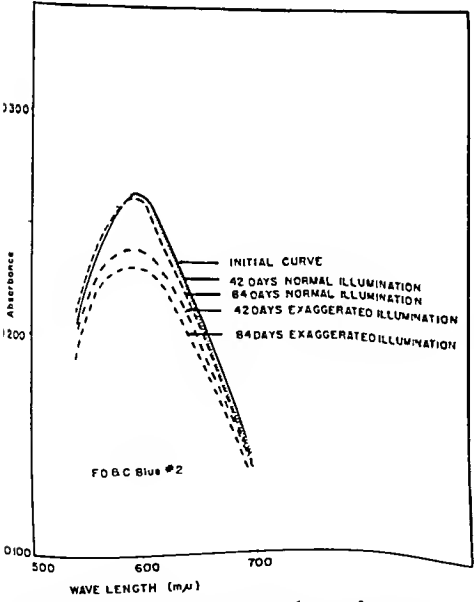


Fig 3—Plots of the visible absorption spectra of D&C Blue No. 2 after different intervals of storage under normal and exaggerated illumination

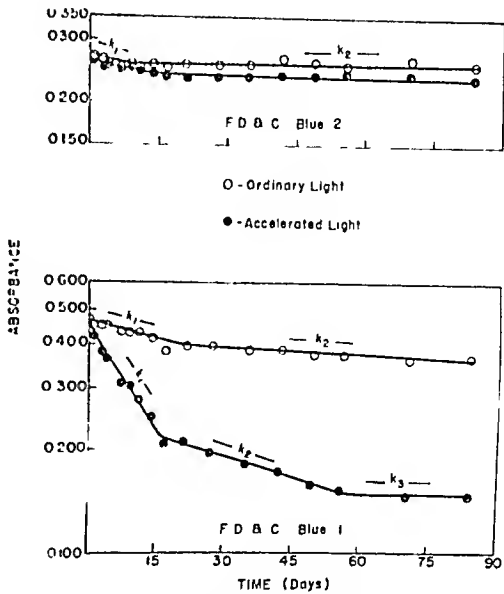


Fig. 4.—Influence of light intensity on the fading of the surfaces of tablets colored with FD&C Blue No. 2 and FD&C Blue No. 1.

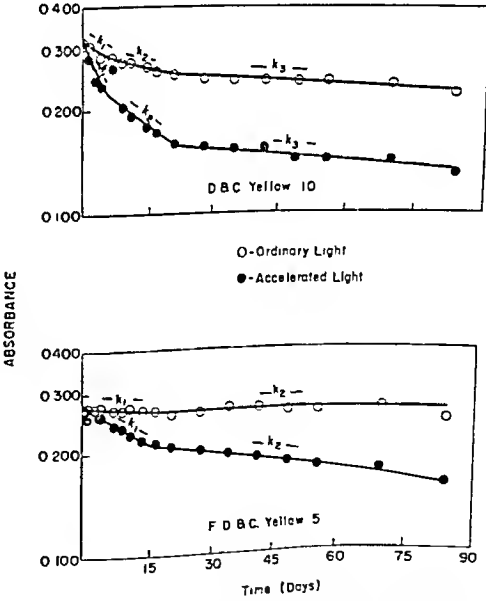


Fig. 5.—Influence of light intensity on the fading of the surface of tablets colored with D&C Yellow No. 10 and FD&C Yellow No. 5.

press, Beckman spectrophotometer model DU with reflectance attachment, die attachment for centering and leveling tablets in reflectance units as described in previous paper of this series (8), precision rotary microtome, and Beckman aquameter.

**Preparation of Tablets.**—Tablets containing the various water-soluble certified dyes were prepared according to the following formula:

Calcium sulfate, dihydrate.....	96.99%
Tragacanth U. S. P.....	2.00%
Magnesium stearate, U. S. P.....	1.00%
Dye.....	0.01%
Ethyl alcohol 50%.....	q. s.

A granulation of the above formula was prepared in accordance with commonly-employed tabletting procedures. The dye was dispersed by dissolving it in the 50% alcohol which was used as a granulating agent. Tablets weighing 700 mg. were compressed using  $16/32$  inch flat punches, uppers bisected. The tablets were all compressed to a hardness of approximately 7 Kg./in.<sup>2</sup> and a thickness of 3 mm. on a Stokes model F tablet press. The moisture content of the dried granulations was kept consistently below 0.5%.

**Storage of Samples in the Light Stability Cabinets.**—Adequate samples of the several differently colored tablets were put into three-inch uncovered clear-glass Petri dishes which were then placed into the normal and intensified light cabinets. The intensity of the light incident on the tablets in the respective cabinets was kept at a constant level through measurement of the light intensity with a Gossen Tri Lux meter at regular time intervals. The light intensities falling on the tablets in the normal and exaggerated illumination cabinets were 45 and 550 foot candles, respectively. Tablet samples were withdrawn from the cabinets at designated time intervals for reflectance measurements.

**Measurement of Tablet Reflectance.**—The tablets removed from the light stability cabinets were measured for fading of the color at the tablet surface by the use of a Beckman model DU spectrophotometer with a modified Beckman reflectance unit attachment to permit its use for single tablets. The equipment modifications and method of measurement are described in detail in a previous publication (8). Reflectance measurements were made at 640  $m\mu$  for FD&C Blue No. 1, at 500  $m\mu$  for FD&C Red No. 1, at 540  $m\mu$  for FD&C Red No. 3, at 620  $m\mu$  for FD&C Green No. 3, at 630  $m\mu$  for D&C Green No. 5, at 420  $m\mu$  for D&C Yellow No. 1, at 430  $m\mu$  for FD&C Yellow No. 5, at 610  $m\mu$  for FD&C Blue No. 2, at 590  $m\mu$  for FD&C Violet No. 1, and at 500  $m\mu$  for D&C Orange No. 3.

**Extent of Fading Beneath Surface of Tablet.**—In order to determine the degree of fading at various distances from the surface of the tablet individual tablets were sliced to definite thicknesses with a microtome. Each tablet was placed in a die which was subsequently leveled in the microtome to permit uniform vertical cuts of the flat surface of the tablet. Reflectance measurements were performed for the several colored tablets at distances up to 300  $\mu$  from the surface.

**Determination of Moisture Content of Tablets.**—Water contained in the tablets was measured by Karl Fischer titration, the end point being determined

electrometrically utilizing the Beckman aquameter. Sample tablets were crushed in a mortar and weighed aliquots removed for dispersion in methanol. This dispersion, under constant stirring, was then titrated with Karl Fischer reagent.

## RESULTS AND DISCUSSION

The photosensitivity of the several dyes used in the tablets studied was determined by exposure to normal and intensified illumination.

Representative plots showing a decrease in the spectral absorbance of the surface of the tablets colored with FD&C Blue No. 1, FD&C Yellow No. 5, and FD&C Blue No. 2 are presented in Figs. 1, 2, and 3. It can be seen from these curves that a substantial variance in the degree of color fading exists, depending upon whether the tablets are exposed under normal or exaggerated illumination. In addition, the rate of fading of the surface color of the tablets is not the same for the several dyes studied, but varies according to the individual color used. An interesting fact brought out by these curves is the change that results in the absorption maximum as the fading progresses. A flattening as well as a hypsochromic shift of the absorption maximum occurs. The magnitude of this effect is greatest for FD&C Blue No. 1 and least for FD&C Blue No. 2, with FD&C Yellow No. 5 falling in between.

Representative plots indicating the rate of fading of dye from the surface of the tablets are given in Figs. 4 and 5. It is readily evident from the linearity of the plots that the fading follows an apparent first-order reaction. These graphs further show the presence of more than one slope for the plots of log absorbance *vs.* time for most of the samples studied. In all cases, the initial rate of fading was greater than that obtained from subsequent portions of the fading curve. Baxter, *et al.* (9), in their studies of the fastness of dyes in textiles also found fading to

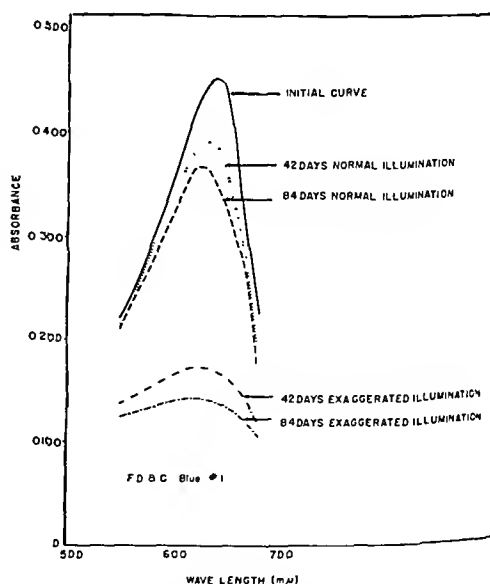


Fig. 1.—Plots of the visible absorption spectra of FD&C Blue No. 1 after different intervals of storage under normal and exaggerated illumination.

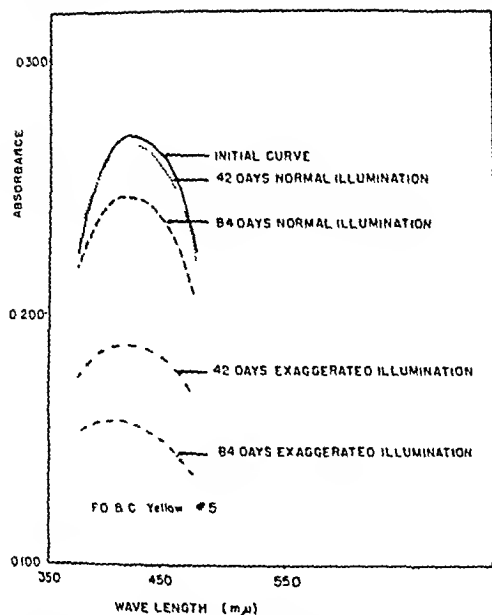


Fig. 2—Plots of the visible absorption spectra of FD&C Yellow No. 5 after different intervals of storage under normal and exaggerated illumination

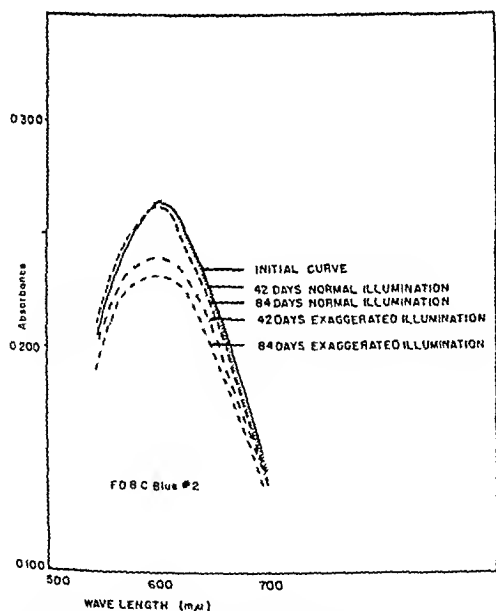


Fig. 3—Plots of the visible absorption spectra of FD&C Blue No. 2 after different intervals of storage under normal and exaggerated illumination.

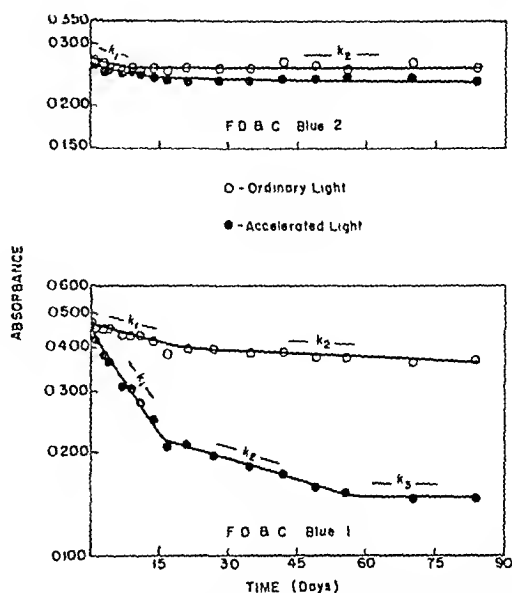


Fig. 4—Influence of light intensity on the fading of the surfaces of tablets colored with FD&C Blue No. 2 and FD&C Blue No. 1

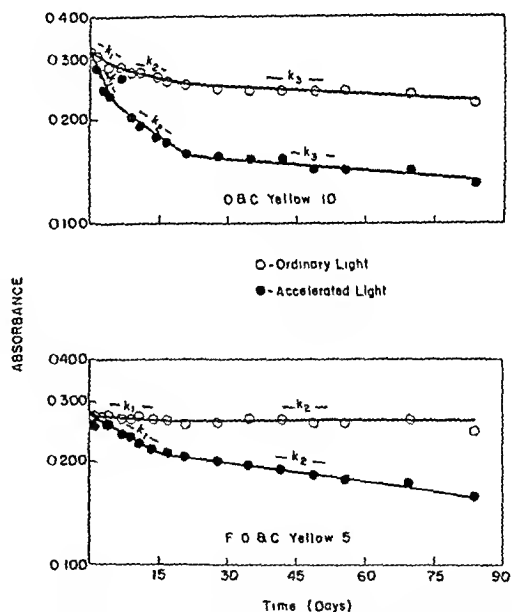


Fig. 5.—Influence of light intensity on the fading of the surface of tablets colored with D&C Yellow No. 10 and FD&C Yellow No. 5.

be much more rapid during the earlier stages of irradiation

The changes in reaction rates that are observed as the duration of irradiation is increased do not necessarily indicate that the fading mechanism consists of a series of consecutive reactions of different rates. Instead, they are most likely due to several contributing factors, among which can be (a) alterations in the quantity of light reflected and absorbed at the

surface of the tablet as fading proceeds, (b) subsequent changes in the amount of energy available to bring molecules to the excited state, (c) possible absorption of a certain amount of energy by the decomposition products, (d) porous nature of the tablets, (e) influence of the substrates on the photoreaction, (f) the possibility of reactions between dye and its decomposition products, (g) secondary reactions involving the dye and atmosphere.

Because it was felt that one or more of the above

factors probably contribute to the changes in the rates observed in the plots of log absorbance *vs.* time it was decided to utilize the initial fading rate as most accurately representing the photodegradation of the dye at the tablet surface. This postulation seems to be supported by Desai and Giles (3) and Atherton and Seltzer (10) in their studies on dye fading. Desai and Giles indicated that the initial reaction is representative of an attack on one or other of the centers of the molecule which are responsible for color. Atherton and Seltzer based their experiments on measurements made over the initial stages of reaction in order to minimize the effect of reactions between the dye and its decomposition products.

The apparent first-order reaction rates for the disappearance of color from the surface of the tablets upon exposure to light were obtained from the slopes of the plots of log absorbance *vs.* time according to the equation,  $\log A_t = -[k/2.303]t + \text{constant}$ , where  $-k/2.303$  is the slope of the plots. The rate constants (*k*) evaluated in this manner are presented in Table I. Although the rate constants for the different slopes of each curve are given, only the initial rate, *k*<sub>1</sub>, will be used in subsequent discussions for reasons previously mentioned.

It is evident from the data presented in Table I that the exaggeration produced by the intensified light source on the rate of fading of the several differently colored tablets is dependent upon the dye used and does not follow any particular pattern. The acceleration in fading rate caused by the exaggerated illumination ranged from 1.5-fold for FD&C Blue No. 2 to 240-fold for D&C Green No. 5, when compared with normal illumination fading.

The contribution of structural configuration of the dye molecule to its photosensitivity is a generally accepted fact in the field of dye chemistry (11). In addition, it is somewhat hazardous to postulate a general relationship between color fastness and structural classification of the dye, i. e., triphenylmethane, azo, indigoid, etc., since it has been shown by Desai and Vaidya (12) that dyes of the same chemical classification vary in fastness to light.

In this study, the dyes used in the tablets exposed to normal illumination exhibit the following decreasing order of light stability: D&C Green No. 5 > D&C Orange No. 3 > FD&C Yellow No. 5 > FD&C Blue No. 2 > FD&C Green No. 3 > FD&C Blue No. 1 > FD&C Violet No. 1 > FD&C Red No. 1 > D&C Yellow No. 10 > FD&C Red No. 3. On the other hand, the tablets exposed to the exaggerated illumination exhibit the following decreasing order of light fastness: FD&C Blue No. 2 > FD&C Yellow No. 5 > D&C Orange No. 3 > FD&C Green No. 3 > FD&C

Blue No. 1 > D&C Green No. 5 > FD&C Red No. 1 > D&C Yellow No. 10 > FD&C Violet No. 1 > FD&C Red No. 3. It is thus apparent that several of the dyes under exaggerated illumination do not possess the same order of relative light fastness as exhibited under normal illumination.

It has been reported by Taylor and Pracejus (6) in their study on the fading of colored plastics and textiles, that due to the porous mesh nature of textiles, depth penetration of all radiations is permitted, subsequently causing color fading at appreciable depths from the surface. Since compressed tablets vary in porosity as a result of the particle size of the granulation as well as the compression force used to prepare them, it is believed that depth fading should also occur in tablets. In order to ascertain this, tablets colored with FD&C Violet No. 1 and exposed to the normal and exaggerated illumination for a period of eighty-four days were microtomed to different depths from the surface. Reflectance measurements were then performed on these new surfaces and the data are presented in Fig. 6.

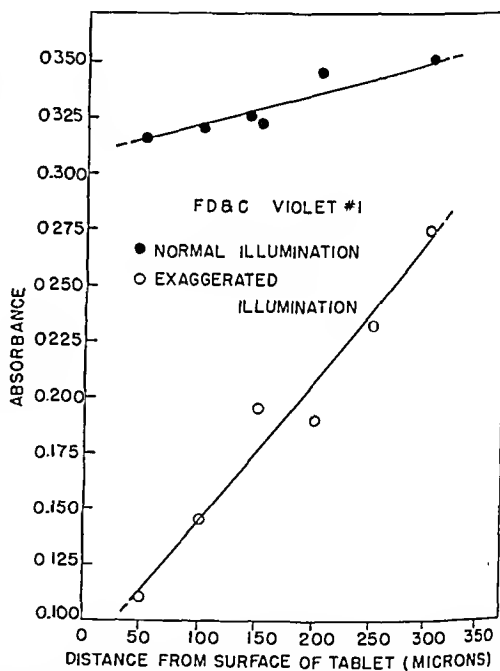


Fig. 6.—A plot showing the extent of fading beneath the surface of tablets colored with FD&C Violet No. 1.

TABLE I.—RATE CONSTANTS FOR THE FADING OF COLORS IN COMPRESSED TABLETS IN DAYS<sup>-1</sup>

Color	Ordinary Lighting			Accelerated Lighting		
	<i>k</i> <sub>1</sub> × 10 <sup>3</sup>	<i>k</i> <sub>2</sub> × 10 <sup>3</sup>	<i>k</i> <sub>3</sub> × 10 <sup>3</sup>	<i>k</i> <sub>1</sub> × 10 <sup>3</sup>	<i>k</i> <sub>2</sub> × 10 <sup>3</sup>	<i>k</i> <sub>3</sub> × 10 <sup>3</sup>
FD&C Red No. 1	15.8	5.11	1.09	69.0	19.5	4.4
FD&C Red No. 3	71.2	22.2	5.9	264.0	25.5	9.2
FD&C Blue No. 1	5.48	1.63	..	50.6	9.4	..
FD&C Blue No. 2	3.45	..	..	5.75	0.15	..
FD&C Green No. 3	4.55	0.75	..	29.2	10.9	4.98
D&C Green No. 5	0.25	..	..	59.8	20.4	1.88
FD&C Yellow No. 5	2.04	0.38	..	16.3	4.78	..
D&C Yellow No. 10	16.4	8.70	1.22	72.1	22.1	3.29
FD&C Violet No. 1	14.6	6.13	2.26	78.0	35.6	8.36
D&C Orange No. 3	0.25	..	..	20.7	2.88	..

It is readily evident from the resulting plots that a substantial amount of fading takes place beneath the surface of the tablet. Furthermore it is interesting to note that by plotting absorbance vs. distance from the surface, straight line plots are obtained for the tablets colored with FD&C Violet No. 1 exposed to normal and exaggerated illumination as evidenced in Fig. 6. Because the differently colored tablets used in this study were prepared from granulations of the same particle size distribution as well as being compressed to the same hardness, they should all exhibit approximately the same porosity. Accordingly, they should also exhibit color fading beneath the surface. Two additional tablets, tablets colored with FD&C Blue No. 1 and D&C Green No. 3, were tested similarly to FD&C Violet No. 1 in order to determine the correctness of this assumption. These two tablets also exhibited significant fading of color beneath the tablet surface.

Inasmuch as fading of colored tablets does not result in a uniform reduction in dye concentration throughout the entire tablet but rather is, to a large extent, a surface phenomenon, it is difficult to correlate extent of fading with the destruction of a certain proportion of the dye initially present in the tablet. Therefore, when the reflectance at the surface of the tablet shows that the dye has faded 50% it does not correspond to 50% total dye destroyed.

The tablet excipients were carefully chosen for this study so that they would not exhibit any significant substrate effect on the fading rate. In addition, the moisture content of the tablets was kept below 0.5% in order to prevent exaggeration of the fading reaction.

Although it may be desirable to use a monochromatic light source for the measurement of dye photosensitivity, a polychromatic source was used in this study in order to simulate practical storage conditions.

## SUMMARY AND CONCLUSIONS

The photosensitivity of ten water-soluble certified dyes in tablets exposed to normal and

exaggerated illumination is evaluated. From the results of this study the following conclusions can be drawn.

1. The absorption curves presented for FD&D Blue No. 1, FD&C Yellow No. 5, and FD&C Blue No. 2 show a hypsochromic shift as well as a flattening of the absorption maximum in the visible spectrum as fading progresses.

2. The apparent degradation rates for the various certified dyes studied are presented and appear to follow a first-order reaction.

3. The fading curves for the dyes investigated show that the rate is most rapid during the initial stage of irradiation.

4. The exaggeration of color fading produced by the intensified illumination does not follow a particular pattern for the dyes studied. The potentiation of the fading rate varies from 1.5- to 240-fold, depending upon the dye studied.

5. Fading beneath the surface of the tablet has been found in the several colored tablets studied. This depth fading can be ascribed to the inherent porosity of compressed tablets.

## REFERENCES

- (1) Blaisdell, B. E., *J. Soc. Dyers Colourists*, **65**, 618 (1949).
- (2) Frauck, R. and Livingston, J., *J. Chem. Phys.*, **9**, 184 (1941).
- (3) Desai, N. F. and Giles, C. H., *J. Soc. Dyers Colourists*, **65**, 635 (1949).
- (4) Weiss, T., *Trans. Faraday Soc.*, **35**, 48 (1939).
- (5) Weiss, T., *ibid.*, **42**, 133 (1946).
- (6) Taylor, A. H., and Pracejus, W. G., *Illum. Eng.*, **45**, 149 (1950).
- (7) Lachman, L., Swartz, C. J., and Cooper, J., *THIS JOURNAL*, in press.
- (8) Urbanyi, T., Swartz, C. J., and Lachman, L., *THIS JOURNAL*, **49**, 163 (1960).
- (9) Baxter, G., Giles, C. H., McKee, M., and Macauley, N., *J. Soc. Dyers Colourists*, **71**, 218 (1955).
- (10) Atherton, E., and Seltzer, I., *ibid.*, **65**, 629 (1949).
- (11) Lubs, H. A., "The Chemistry of Synthetic Dyes and Pigments," Reinhold Publishing Corp., New York, N. Y., 1955, pp. 622-686.
- (12) Desai, C. M., and Vaidya, B. K., *J. Indian Chem. Soc.*, **31**, 261, 389 (1954).

# Serotonin-Induced Apnea\*

By D. C. KROEGER and L. J. LUCCO†

Studies have been conducted on a number of species of animals to determine the respective role of peripheral and central actions of serotonin which causes apnea. In dogs, three respiratory events are obtained with i. v. injections of serotonin; transient gasping and a slower developing hyperpnea which is interrupted in a majority of the dogs by apnea. Both forms of respiratory stimulation are sensitive to procaine endoanesthesia, gasping being selectively blocked by carotid sinus denervation. Hypercapemia resulting from apnea and bronchoconstriction play a role in the hyperpnea. Apnea in the dog is of a different origin than that in the cat. Preventing bronchoconstriction reduces the occurrence of apnea in the dog. Evidence is presented for a central (medullary) inhibition of reflex gasping by serotonin.

**S**PECIES DIFFERENCES to the effects of serotonin, 5-hydroxytryptamine (5-HT) are well known (1). Cats and dogs show opposite respiratory responses to intravenous injections of 5-HT. In cats the usual response is apnea which is followed by hyperpnea, whereas in the dog, apnea is frequently seen to interrupt an existing hyperpnea.

Currently many investigators and reviewers attribute the apnea to a reflex originating from the stimulation of receptors lying in the cardiopulmonary circulation circuit (2-6) and the hyperpnea to a reflex arising from stimulation of receptors in the aorto carotid baro- and chemoreceptive areas (7-9). The situation is much confused, however, for not only does 5-HT stimulate peripheral receptors (10), but exerts a direct effect upon ganglionic transmission (11), smooth muscle (12), and central nervous system (13), and also releases histamine into the general circulation (14).

A survey of the literature suggests, however, that some of the current confusion regarding the respiratory actions of 5-HT lies in the fact that certain phenomena involved with respiration have not been considered. The terms respiratory stimulation, hyperpnea, and apnea have been used with little clarification as to whether these consisted of changes in rate, amplitude, duration and/or even changes in the type of respiration. Thus, the purpose of this study is to define certain respiratory characteristics of 5-HT, to present evidence for some possible mechanisms of actions, and to review the pertinent literature in view of these findings.

## EXPERIMENTAL

**Methods.**—Forty-five dogs weighing 8-16 Kg, 15 cats, 2-3 Kg, two rhesus monkeys, 2-3 Kg, and three chickens weighing 0.5 Kg were used in this study. Various anesthetic mixtures were used including i. v. pentobarbital, 30 mg/Kg, pentobarbital-morphine, 10 mg each/Kg, and chloralose, 75-90 mg/Kg.

For routine experiments, blood pressure was recorded from the femoral artery with a Statham or E+M blood pressure transducer. Respiration in dogs and certain of the other animals was recorded using an intrathoracic trocar attached to a Statham transducer, otherwise, chest movements were recorded using an E+M stethograph. After tracheal cannulation, respiratory exchange was measured with a Benedict-Roth spirometer and continuous blood oxygen levels monitored with an E+M flow-through oximeter connected to the carotid circulation. Carotid and jugular blood samples were simultaneously obtained anaerobically and analyzed for blood gases by the Van Slyke method. The carotid bifurcations were exposed and the carotid sinus and carotid body nerves isolated and identified using the protocol outlined by Neil, *et al* (15). The majority of injections were administered rapidly into the femoral vein with a saline wash, other injections were made into the carotids and jugulars. Sodium mannuronate, 10 mg/Kg, was used to prevent clotting.

Special procedures for six cats consisted of bilateral isolation of the carotid sinus and body with their nerves. The floor of the fourth ventricle was exposed by removal of the cerebellum and evoked potentials recorded using insulated nichrome electrodes RC coupled to a dual-beam oscilloscope. Electrode placement and microinjection of drugs into the medulla were made using a Lab-Tronic stereotaxic apparatus. Carotid blood pressure was recorded with a Hathaway transducer and written out on the Physiograph<sup>1</sup> as was all other transduced data. Blood pressure was maintained by slow i. v. infusion of norepinephrine and body temperature held near normal with a heating pad.

Doses of 5-HT are listed as mcg 5-HT base/Kg of body weight. Two different samples of serotonin creatinine sulfate were available for this study (Abbott Laboratories and California Foundation

\* Received August 21, 1959 from the Pharmacology Laboratories, Dental Branch, University of Texas, Houston 25. Data concerning blood gas analyses is abstracted from a thesis submitted to the Graduate School of the University of Houston by L. J. Lucco in partial fulfillment of the requirements for the Master of Science degree.

† Present address: Veterans Administration Hospital, Houston, Tex.

Presented to the Scientific Section A, P.H.A., Cincinnati meeting, August 1959.

<sup>1</sup> Manufactured by the I + M Instrument Company, Houston, Tex.

for Biochemical Research); however, no difference in activity was noted. Except where noted, at least fifteen minutes was allowed between repeat injections of 5-HT. Other drugs used in this study were procaine hydrochloride, bufotenine monooxylate (California Foundation for Biochemical Research), lysergic acid diethylamide (Sandoz, Delysid), reserpine phosphate (Ciba), and (*n*-ethyl-3-piperidyl)-phenylethyl glycolate (Lakeside J.B. 329).

**Results.**—Rapid injection of doses of 5-HT from 1 to 100 mcg./Kg. were given into the femoral vein of dogs. The latency for the initial sign of a respiratory change was  $6.04 \pm 1.8$  seconds. Bradycardia was usually seen simultaneously with the occurrence of the respiratory change. Immediately after the initial hyperventilation, four response patterns were observed (in the order of decreasing occurrence): a period of respiratory arrest; a decrease in the respiratory rate of amplitude with regard to the control value; continuation of the increased respiratory activity, only of less intensity than that of the initial response; a period in which the respiratory rate and amplitude rapidly returned to the control value. Generally the same response continued to be shown in the same dog with repeated injections.

The usual response obtained is shown in Fig. 1. With the injection of 30 mcg./Kg. of 5-HT there is a brief period averaging  $12.1 \pm 3.8$  seconds of extreme respiratory effort, not only has the rate increased an average of  $4.7 \pm 1.6$  times but there is an increase in the expiratory as well as the inspiratory air. Following this period of both tachypnea and hyperpnea there begins a period of respiratory arrest in the expiratory stage. Negative pressure in the thoracic cavity during the arrest period is slightly less than the control period, indicating a relaxation of the muscles about the thoracic cage. Following the period of complete arrest the respiratory muscles are again contracted to a much greater extent than normal but at a much slower rate than the control. Within a minute's time the rate has increased to slightly more than control and the amplitude of inspiration has decreased but is still approximately twice the control. The respiratory picture slowly declines both in amplitude and rate until it is back to control levels in five to ten minutes.

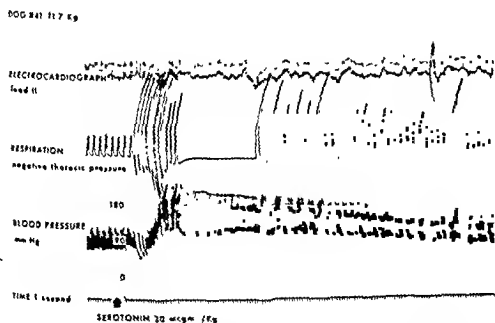


Fig. 1.—Dog 11.7 Kg. male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injection of 5-HT on electrocardiogram, respiration, and blood pressure in the intact anesthetized dog. Electrocardiograph; lead II, respiration; intrathoracic pressure, inspiration up. Blood pressure in mm. Hg. Time intervals are one second and one minute.

Figure 2 is a similar trace upon which we have indicated certain theoretical phenomena. The three phases represented here compare favorably with the three phases of 5-HT action described by Schneider and Rinehart (16). In this tracing there first appears inspiratory efforts which resemble and have been labeled as gasps. Shortly following the initial gasp there begins a secondary type of respiratory stimulation which involves not only an increase in the inspiratory effort but also forced expiration. Both of these events are suddenly terminated by respiratory arrest with the intrathoracic pressure again somewhat greater than during the control period. The inhibition of inspiration is terminated first but is shortly followed by the reappearance of a forced expiration. At this dose the hyperpnea, but not tachypnea, continues for an additional five minutes.

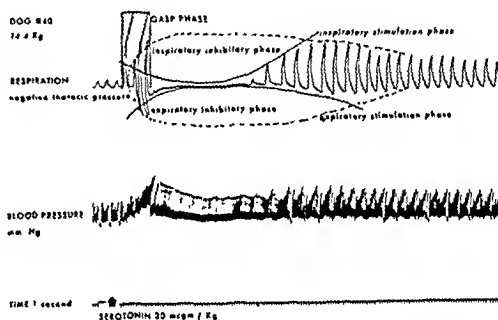


Fig. 2.—Dog 14.4 Kg., male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injection of 5-HT on respiration and blood pressure in the intact anesthetized dog. Respiration, intrathoracic pressure, inspiration up. Blood pressure in mm. Hg. Time intervals are one second and one minute. Three phases of the respiratory reaction are designated; phase 1 (gasp phase), phase 2 (combined inspiratory and expiratory excitatory phase), and phase 3 (combined inspiratory and expiratory inhibitory phase).

Another reaction which would influence the latter phase of respiratory stimulation is seen in Fig. 3. With the injection of 30 mcg./Kg. 5-HT, the increase in the depth and rate of respiration causes the oxygen saturation to increase briefly but it then begins to fall dramatically even though the respiration appears to be more than adequate. During the inhibitory phase there are a few aborted inspirations. The spirometer trace, however, indicates a lack of tidal movement in spite of rather rapid and excessive respiratory movements. The lack of gaseous exchange would appear largely due to bronchoconstriction rather than pulmonary vasoconstriction since the blood pressure tracing does not indicate an interference in cardiac output due to poor filling of the left heart. These findings confirm those of Konzett (17). Recent data by Borst, *et al.* (18), however, indicates a considerable pulmonary vasoconstriction by 5-HT and perhaps the lung by-pass via pulmonary A-V fistulas described by Sparks and Tombridge (19) might play a role. Reid and Rand (20), Comroe, *et al.* (2), and Broeklehurst (12) have demonstrated considerable bronchoconstriction.

A series of 12 dogs were studied with respect to blood gases following i. v. injection of 5-HT, 13



# Serotonin-Induced Apnea\*

By D. C. KROEGER and L. J. LUCCO†

Studies have been conducted on a number of species of animals to determine the respective role of peripheral and central actions of serotonin which causes apnea. In dogs, three respiratory events are obtained with i. v. injections of serotonin; transient gasping and a slower developing hyperpnea which is interrupted in a majority of the dogs by apnea. Both forms of respiratory stimulation are sensitive to procaine endoanesthesia, gasping being selectively blocked by carotid sinus denervation. Hypercapemia resulting from apnea and bronchoconstriction play a role in the hyperpnea. Apnea in the dog is of a different origin than that in the cat. Preventing bronchoconstriction reduces the occurrence of apnea in the dog. Evidence is presented for a central (medullary) inhibition of reflex gasping by serotonin.

**S**PECIES DIFFERENCES to the effects of serotonin, 5-hydroxytryptamine (5-HT) are well known (1). Cats and dogs show opposite respiratory responses to intravenous injections of 5-HT. In cats the usual response is apnea which is followed by hyperpnea, whereas in the dog, apnea is frequently seen to interrupt an existing hyperpnea.

Currently many investigators and reviewers attribute the apnea to a reflex originating from the stimulation of receptors lying in the cardiopulmonary circulation circuit (2-6) and the hyperpnea to a reflex arising from stimulation of receptors in the aorto-carotid baro- and chemoreceptive areas (7-9). The situation is much confused, however, for not only does 5-HT stimulate peripheral receptors (10), but exerts a direct effect upon ganglionic transmission (11), smooth muscle (12), and central nervous system (13), and also releases histamine into the general circulation (14).

A survey of the literature suggests, however, that some of the current confusion regarding the respiratory actions of 5-HT lies in the fact that certain phenomena involved with respiration have not been considered. The terms respiratory stimulation, hyperpnea, and apnea have been used with little clarification as to whether these consisted of changes in rate, amplitude, duration and/or even changes in the type of respiration. Thus, the purpose of this study is to define certain respiratory characteristics of 5-HT, to present evidence for some possible mechanisms of actions, and to review the pertinent literature in view of these findings.

## EXPERIMENTAL

**Methods.**—Forty-five dogs weighing 8-16 Kg; 15 cats, 2-3 Kg; two rhesus monkeys, 2-3 Kg.; and three chickens weighing 0.5 Kg. were used in this study. Various anesthetic mixtures were used including i. v. pentobarbital, 30 mg./Kg.; pentobarbital-morphine, 10 mg. each/Kg.; and chloralose, 75-90 mg./Kg.

For routine experiments, blood pressure was recorded from the femoral artery with a Statham or E+M blood pressure transducer. Respiration in dogs and certain of the other animals was recorded using an intrathoracic trochar attached to a Statham transducer, otherwise, chest movements were recorded using an E+M stethograph. After tracheal cannulation, respiratory exchange was measured with a Benedict-Roth spirometer and continuous blood oxygen levels monitored with an E+M flow-through oximeter connected to the carotid circulation. Carotid and jugular blood samples were simultaneously obtained anaerobically and analyzed for blood gases by the Van Slyke method. The carotid bifurcations were exposed and the carotid sinus and carotid body nerves isolated and identified using the protocol outlined by Neil, *et al.* (15). The majority of injections were administered rapidly into the femoral vein with a saline wash, other injections were made into the carotids and jugulars. Sodium mannuronate, 10 mg/Kg., was used to prevent clotting.

Special procedures for six cats consisted of bilateral isolation of the carotid sinus and body with their nerves. The floor of the fourth ventricle was exposed by removal of the cerebellum and evoked potentials recorded using insulated nichrome electrodes RC coupled to a dual-beam oscilloscope. Electrode placement and microinjection of drugs into the medulla were made using a Lab-Tronic stereotaxic apparatus. Carotid blood pressure was recorded with a Hathaway transducer and written out on the Physiograph<sup>1</sup> as was all other transduced data. Blood pressure was maintained by slow i. v. infusion of norepinephrine and body temperature held near normal with a heating pad.

Doses of 5-HT are listed as mcg. 5-HT base/Kg. of body weight. Two different samples of serotonin creatinine sulfate were available for this study (Abbott Laboratories and California Foundation

\* Received August 21, 1959, from the Pharmacology Laboratories, Dental Branch, University of Texas, Houston 25  
† Data concerning blood gas analyses is abstracted from a thesis submitted to the Graduate School of the University of Houston by L. J. Lucco in partial fulfillment of the requirements for the Master of Science degree

† Present address: Veterans Administration Hospital, Houston, Tex  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

<sup>1</sup> Manufactured by the E+M Instrument Company, Houston, Tex.

for Biochemical Research), however, no difference in activity was noted. Except where noted at least fifteen minutes was allowed between repeat injections of 5-HT. Other drugs used in this study were procaine hydrochloride, bufotenine monohydrate (California Foundation for Biochemical Research), l-tryptophan diethylamide (Sandoz, Delvid), reserpine phosphate (Ciba), and (n-ethyl 3 piperidyl) phenylethanol glycolate (Lakeside J B 329).

**Results**—Rapid injection of doses of 5-HT from 1 to 100 mg/Kg were given into the femoral vein of dogs. The latency for the initial sign of a respiratory change was  $6.04 \pm 1.8$  seconds. Bradycardia was usually seen simultaneously with the occurrence of the respiratory change. Immediately after the initial hyperpnea, four response patterns were observed (in the order of decreasing occurrence): a period of respiratory arrest, a decrease in the respiratory rate of amplitude with regard to the control value, continuation of the increased respiratory activity, only of less intensity than that of the initial response, a period in which the respiratory rate and amplitude rapidly returned to the control value. Generally the same response continued to be shown in the same dog with repeated injections.

The usual response obtained is shown in Fig 1. With the injection of 30 mg/Kg of 5-HT there is a brief period averaging  $12.1 \pm 3.8$  seconds of extreme respiratory effort, not only has the rate increased an average of  $4.7 \pm 1.6$  times but there is an increase in the expiratory as well as the inspiratory air. Following this period of both tachypnea and hyperpnea there begins a period of respiratory arrest in the expiratory stage. Negative pressure in the thoracic cavity during the arrest period is slightly less than the control period, indicating a relaxation of the muscles about the thoracic cage. Following the period of complete arrest the respiratory muscles are again contracted to a much greater extent than normal but at a much slower rate than the control. Within a minute's time the rate has increased to slightly more than control and the amplitude of inspiration has decreased but is still approximately twice the control. The respiratory picture slowly declines both in amplitude and rate until it is back to control levels in five to ten minutes.

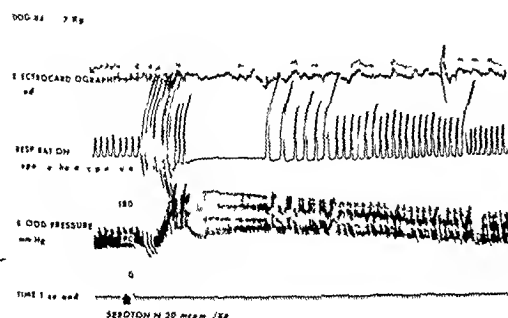


Fig 1—Dog 117 Kg, male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injection of 5-HT on electrocardiogram, respiration, and blood pressure in the intact anesthetized dog. Electrocardiograph, lead II, respiration, intrathoracic pressure, inspiration up. Blood pressure in mm Hg. Time intervals are one second and one minute.

Figure 2 is a similar trace upon which we have indicated certain theoretical phenomena. The three phases represented here compare favorably with the three phases of 5-HT action described by Schneider and Rinehart (16). In this tracing there first appears inspiratory efforts which resemble and have been labeled as gasps. Shortly following the initial gasp there begins a secondary type of respiratory stimulation which involves not only an increase in the inspiratory effort but also forced expiration. Both of these events are suddenly terminated by respiratory arrest with the intrathoracic pressure again somewhat greater than during the control period. The inhibition of inspiration is terminated first but is shortly followed by the reappearance of a forced expiration. At this dose the hyperpnea, but not tachypnea, continues for an additional five minutes.

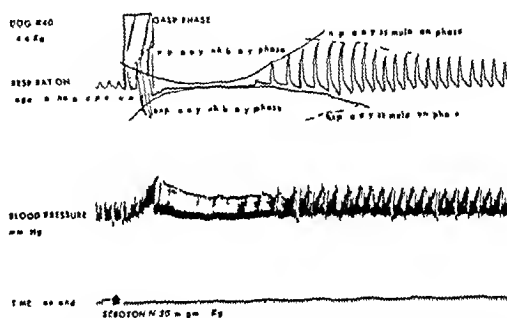


Fig 2—Dog 144 Kg, male. Anesthetic, pentobarbital-morphine. Effect of single intravenous injection of 5-HT on respiration and blood pressure in the intact anesthetized dog. Respiration, intrathoracic pressure, inspiration up. Blood pressure in mm Hg. Time intervals are one second and one minute. Three phases of the respiratory reaction are designated, phase 1 (gasp phase), phase 2 (combined inspiratory and expiratory excitatory phase), and phase 3 (combined inspiratory and expiratory inhibitory phase).

Another reaction which would influence the latter phase of respiratory stimulation is seen in Fig 3. With the injection of 30 mg/Kg 5-HT, the increase in the depth and rate of respiration causes the oxygen saturation to increase briefly but it then begins to fall dramatically even though the respiration appears to be more than adequate. During the inhibitory phase there are a few aborted inspirations. The spirometer trace, however, indicates a lack of tidal movement in spite of rather rapid and excessive respiratory movements. The lack of gaseous exchange would appear largely due to bronchoconstriction rather than pulmonary vasoconstriction since the blood pressure tracing does not indicate an interference in cardiac output due to poor filling of the left heart. These findings confirm those of Konzett (17). Recent data by Borst, *et al* (18), however, indicates a considerable pulmonary vasoconstriction by 5-HT and perhaps the lung by-pass via pulmonary A-V fistulas described by Sparks and Tombridge (19) might play a role. Reid and Rand (20), Comroe, *et al* (2), and Brocklehurst (12) have demonstrated considerable bronchoconstriction.

A series of 12 dogs were studied with respect to blood gases following i.v. injection of 5-HT, 13

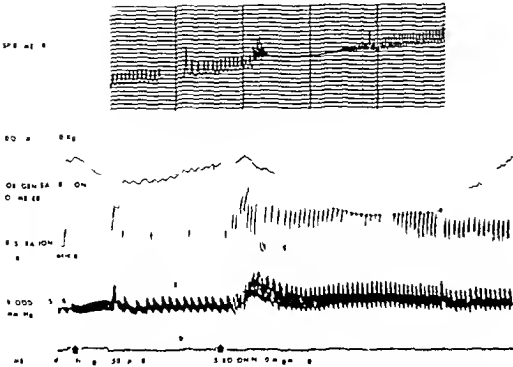


Fig 3—Dog 140 Kg, male Anesthetic, pento barbital morphine Effect of single intravenous injection of 5 HT on respiration and blood pressure in the intact anesthetized dog Top trace is tidal air recorded on a spirometer, vertical lines are one minute time intervals Second trace is carotid artery oxygen saturation Third trace is intrathoracic pressure, inspiration up Spirometer trace is not enlarged to the same scale as Physiograph trace Blood pressure in mm Hg Time intervals are one second and one minute Note the loss of tidal air volumes which coincide with a fall in carotid artery oxygen saturation in spite of continued respiratory movements

mcg /Kg Three doses of 5 HT were administered forty five minutes apart and carotid and jugular blood samples collected just prior to the administration of 5 HT and immediately after termination of the initial respiratory stimulation In this study at this dosage, only 4 of the 12 dogs showed apnea A comparison of the mean oxygen and carbon dioxide contents of the samples pre and postinjection showed a slight increase in the volume per cent of oxygen which was confirmed in the studies using the flow-through oximeter Volume per cent of carbon dioxide showed a decrease Due to individual variations, analysis of variance indicated little treatment significance Table I is a summary of the blood gas analyses of the four animals showing apnea

That this secondary respiratory stimulation is not entirely caused by hypoxia and/or hypercapnia is illustrated in Figs 4 and 5 Figure 4 illustrates the

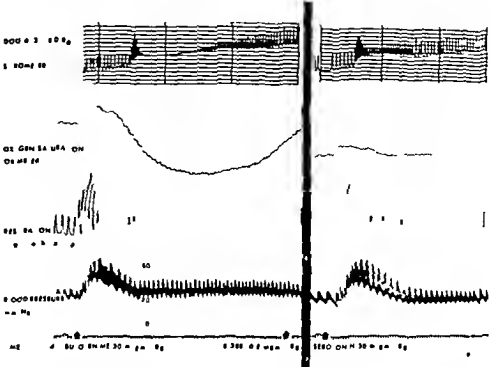


Fig 4—Dog 140 Kg, male Anesthetic, pento barbital-morphine Effect of single intravenous injections of bufotenine and 5 HT on respiration and blood pressure in the intact anesthetized dog Both drugs were given before and after JB 329, one trace of each is presented Similar reactions to both bufotenine and 5-HT were obtained Top trace is the tidal air recorded on a spirometer, vertical lines are one minute intervals Second trace is carotid artery oxygen saturation Third trace is intrathoracic pressure, inspiration up Lower trace is blood pressure in mm Hg Time intervals are one second and one minute Note only partial reduction of tidal air volumes and lack of apnea following injection of JB 329

action of both bufotenine, N-methyl 5 hydroxytryptamine and 5 HT which are nearly identical Bufotenine is capable of causing a strong bronchoconstriction and a cessation of tidal movement of air However, fifteen minutes after the injection of J B 329, 0.2 mcg /Kg, an anticholinergic compound, the bronchoconstriction is greatly reduced This drug also blocks the apneic stage without influencing either the initial gasp or the secondary hyperpnea Reserpine, 0.5 mg /Kg and L S D 25, 25 mcg /Kg, were also capable of blocking the bronchoconstriction but reserpine did not alter the respiratory events This action of reserpine had previously been noticed by Schneider and Rinchart (16)

Figure 5 illustrates the progressive increase in the amount of bronchoconstriction with doses of 10, 20,

TABLE I—OXYGEN AND CARBON DIOXIDE CONTENTS OF ARTERIAL AND VENOUS BLOOD IN DOGS BEFORE AND AFTER THE ADMINISTRATION OF SEROTONIN<sup>a</sup>

Dog No	Wt, Kg	Inj No	Arterial Blood					Venous Blood						
			Oxygen		Change %	Carbon Dioxide		Change %	Oxygen		Change %	Carbon Dioxide		
			Before Inj Vol %	After Inj Vol %		Before Inj Vol %	After Inj Vol %		Before Inj Vol %	After Inj Vol %		Before Inj Vol %	After Inj Vol %	
2	10 65	1 <sup>b</sup>	16 22	18 06	1 13	45 09	42 83	-0 50	14 78	14 97	0 13	47 55	45 91	-0 34
		2 <sup>b</sup>	16 17	17 10	0 58	42 27	39 66	-0 62	15 19	11 68	-2 31	47 79	15 52	-0 48
		3 <sup>b</sup>	16 15	18 51	1 48	44 47	39 47	-1 12	11 84	12 83	0 84	44 18	45 59	0 32
3	11 25	1	17 79	15 42	-1 33	48 97	39 07	-2 02	15 28	21 68	4 19	52 76	50 50	-0 43
		2	18 44	19 31	0 47	45 55	43 67	-0 41	16 80	17 82	0 61	51 02	50 50	-0 10
		3 <sup>b</sup>	17 33	20 66	1 92	38 51	45 28	1 76	15 42	1 73	-6 93	49 51	55 17	1 11
10	7 48	1 <sup>b</sup>	13 04	13 75	0 54	42 43	40 98	-0 34	6 46	7 28	1 27	17 90	17 38	-0 11
		2 <sup>b</sup>	14 04	14 47	0 31	42 00	38 88	-0 71	5 73	6 75	1 78	46 75	16 23	-0 11
		3 <sup>b</sup>	13 31	14 47	0 87	42 00	41 74	-0 06	6 22	6 75	0 85	15 92	39 92	-1 31
11	10 48	1	13 95	14 86	0 55	39 30	38 35	-0 24	9 62	9 08	-0 56	14 07	13 97	-0 02
		2 <sup>b</sup>	15 89	15 46	-0 27	44 60	36 01	0 42	6 00	5 72	-0 17	41 88	43 50	0 39
		3 <sup>b</sup>	14 18	13 47	-0 50	32 10	31 20	0 55	4 71	5 77	2 25	11 01	11 18	0 10

<sup>a</sup> The animals included in this table are only those that demonstrated a distinct apneic response following the initial hyperventilation elicited by the administration of serotonin base (13 mcg /Kg, i v). All percentage changes are positive unless noted  
<sup>b</sup> Injections in which apnea was observed

DOG #44 16.8 Kg.

SPIROMETER

OXYGEN CONSUMPTION

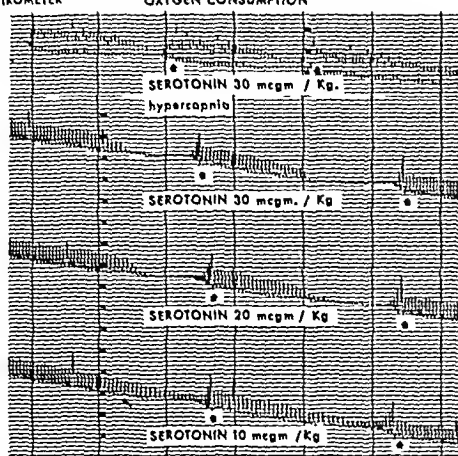


Fig. 5.—Dog 16.8 Kg., male. Anesthetic, pento-barbital-morphine. Effect of various intravenous doses of 5-HT on tidal air and oxygen consumption in the intact anesthetized dog. Spirometer traces read from right to left. Top trace illustrates the effect of increasing the carbon dioxide tension by removal of the soda-lime cannister

and 30 mcg./Kg. of 5-HT. Though these doses were repeated within three minutes there was no apparent desensitization or tachyphylaxis of this bronchoconstriction. The top tracing of Fig 5 shows the lessening of the bronchoconstriction when the animal was made hypercapemic by removal of the soda-lime absorbent from the spirometer filled with 100%  $O_2$ .

Contrary to the report by Schneider and Yonkman (21), procaine 3 mg./Kg. is capable of blocking the initial reflex respiratory stimulation in dogs. With an injection of 5-HT, one to three minutes following the i. v. injection of procaine, the dog shows respiratory arrest or partial inhibition immediately without the respiratory stimulation.

Figure 6 illustrates the reflex gasp still obtained after double cervical vagotomy. Procaine HCl, 5 mg./Kg. i. v., however, abolishes this residual action of 5-HT, 30 mcg./Kg. Sectioning of the vagi abolishes the apneic response to small or moderate dosages of 5-HT; however, if the dose is increased up to 100 mcg. or more/Kg. i. v. in the vagotomized animal, another apneic response is obtained.

To investigate this action further a series of four cats with exposure of the medulla and the carotid bifurcation were employed. Decerebrate cats were made apneic by threshold tetanizing stimulation to the expiratory center on the floor of the medulla rostral to the obex. Injections of 30–60 mcg./Kg. 5-HT i. v. did not consistently block or lessen expiratory center stimulation; however, occasionally an inspiration did occur within the expected latency after injection. Complete apnea still persisted after carotid injections of serotonin. Pontine sectioned cats were made to inspire by intermittent tetanizing stimulation of the inspiratory center. Again injections of 5-HT, 30–60 mcg./Kg. i. v. did not block this form of artificial respiration, nor did microinjections of 0.005 ml. of 0.1% 5-HT at the site of

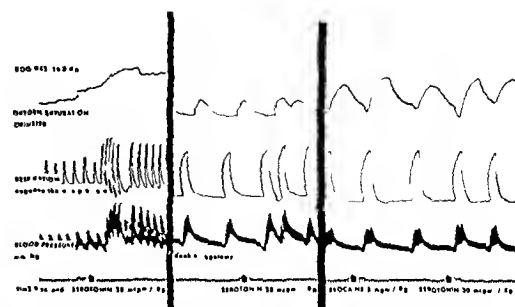


Fig. 6.—Dog 14.0 Kg., male. Anesthetic, pento-barbital-morphine. Effect of intravenous injections of 5-HT on carotid artery oxygen saturation, respiration; intrathoracic pressure and blood pressure in the intact and vagotomized dog. The tracings show the occurrence of a respiratory gasp in the vagotomized dog and blockage by intravenous procaine.

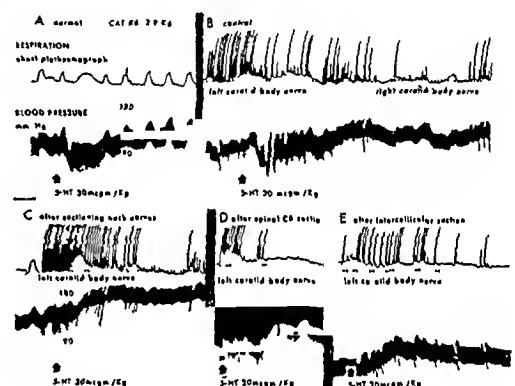


Fig. 7.—Cat 2.9 Kg., female. Anesthetic, chloralose. Effect of intravenous injections of 5-HT on the reflex gasping caused by intermittent tetanizing stimulation of the right or left carotid body nerve. Part A illustrates control responses to 5-HT. Part B illustrates inhibition of gasping by 5-HT in the intact cat. Part C illustrates the same response after sectioning of the vagi and the sympathetic chain. Part D shows a similar effect still present after spinal cord  $C_6$  section. Part E shows this same effect after intercollicular sectioning of the brain stem. Dots appearing under respiration indicate frequency and duration of stimulation

medullary stimulation, but this effect could be blocked by microinjection of procaine 4%.

Figure 7 shows a series of tracings made from a cat which was caused to gasp by intermittent threshold tetanizing stimulation of either the left or right carotid body nerve. With injections of 5-HT, 30 mcg./Kg., this reflex gasp is seen in Fig. 7B to fatigue quite rapidly at the maximum of the depressor response. Switching from the left to the right carotid body nerve indicates that the bilateral reflex center rather than the nerve fiber is involved. The usual reflex gasps are either partially blocked in amplitude or completely prevented.

With the sectioning of all major nerves in the neck, the ganglionic chain below the superior cervical ganglia and the vago-sympathetic network, the blood pressure trace changes from a depressor to a

pressor reaction, Fig. 7C. With the injection of 5-HT, 30 mcg./Kg. i. v., the reflex gasps become inhibited at the maximum of the pressor response. In order to determine that this effect was not due to impulses conducted centrally by the neck nerves or in the spinal cord, a  $C_6$  section was performed. Figure 7D shows the similar effect of the same dose of 5-HT on the induced reflex gasps thirty minutes following cord section. Finally in Fig. 7E, following sectioning of the brain stem above the medulla, the reflex gasps are inhibited coincidentally with the injection of 5-HT. The head of the animal at this stage was connected to the trunk only by the carotid and vertebral arteries, the jugular vein, and the vertebral muscles. All other tissue in the neck had been ligated and severed, including both carotid sinus nerves, thus the only functional tissue would seem to be the cervical spinal cord and medulla connected efferently to the phrenic nerve. The afferent connections, the carotid body nerves, were spared since they were being stimulated for the gasp reflex.

## DISCUSSION

It has been stated previously that some of the confusion existing about the respiratory actions of 5-HT could be attributed to the lack of uniformity of description of the respiratory events. By not fully describing the respiratory reactions seen some of the more subtle actions of 5-HT have been overlooked. Using Fig. 2 upon which the three respiratory phases of 5-HT have been depicted it is possible to review previous findings in the light of some of the results presented in this paper.

The initial stimulation is the only respiratory action of 5-HT that is peculiar to the dog and not to cat, rabbit, rat, and guinea pig. Schneider and Yonkman (4) reported that double vagotomy abolished this primary respiratory stimulation by 5-HT and that the reflex receptor sites must then lie in the cardio-pulmonary region. Douglas and Toh (9), McCubbin, *et al.* (7), however, presented evidence that the origin of the respiratory stimulation is still functional in the vagotomized dog. McCubbin, *et al.*, disagree with Douglas and Toh, Heymans and van den Heuvel-Heymans (22) regarding the role of the carotid sinus and/or the carotid body. The latter two groups of investigators favor a central stimulatory action since stimulation persisted after sinus nerve sectioning in the vagotomized dog.

The same discrepancies and confusion exist for the primary apneic response seen in the cat. Reid and Rand (20) first reported that vagotomy eliminated this response. Comroe, *et al.* (2), Mott and Paintal (5), Paintal (6), Kottegod and Mott (23), and Schneider and Yonkman (4) have all presented evidence that afferent fibers in the vagus are stimulated and sectioning or cooling the vagus to 1 or 2° abolishes apnea in most cats. Ginzel and Kottegod (24) obtained evidence that baro- or chemoreceptive stimulation caused an apnea which was abolished by sinus nerve sectioning. More recently Ginzel (10) has described apnea which persists after sinus nerve sectioning and can be elicited by the injection of small amounts of 5-HT into the subclavian artery near the vertebral artery.

Thus, for both the dog and the cat evidence has been obtained that 5-HT causes respiratory reac-

tions at three different sites, cardio-pulmonary, sino-aortic, and central. As stated earlier, apnea in the cat is predominantly caused by a reflex arising from the lungs, the fibers having been identified by Paintal (6) as specific pulmonary deflator fibers. The initial gasps in dogs would appear largely due to stimulation of the chemoreceptors of sino-aortic origin. It is proposed, however, that coincidental with the gasps there begins a true hyperpnea which may or may not be interrupted by apnea.

That two simultaneous forms of respiratory stimulation exist is suggested by the latent appearance of tachypnea with active expiration following one or more gasps. Inspection of published records (7, Fig. 3) of McCubbin, *et al.*, reveals a tachypnea but without a change in amplitude during which time the chemoreceptors of the carotid bifurcation were removed from contact with circulating 5-HT.

The apneic response in dogs appears to have a dose-response relationship, for Douglas and Toh (9) report no apnea with 2-8 mcg./Kg. i. v. but Lucco (25) obtained a 20% response with 13 mcg./Kg. i. v.; Schneider and Yonkman (21) a 50% response with 50 mcg./Kg. i. v.; and MacCanon and Horvath (8) report a 75% response when 2.5-5.0 mcg./Kg. was injected directly into the pulmonary artery.

Apnea in dogs appears to have an origin other than that seen in the cat. Intravenous injection of procaine prior to 5-HT abolishes apnea in the cat but not in dogs. Procaine does block the initial gasping and hyperpnea seen in the dog. In certain dogs there is close agreement between the dose-response and the time duration for both apnea and bronchoconstriction. The delayed appearance of apnea in dogs also suggests some other mechanism than that proposed for the cat. Preventing bronchoconstriction in certain dogs blocks the apneic response, Fig. 4. Schneider and Rinehart (16) although presenting no data regarding bronchoconstriction, describe the ability of reserpine to partially decrease apnea with better oxygenation. We have been able to repeat this experiment showing that reserpine has prevented bronchoconstriction. Prolongation of respiratory apnea in dogs following ganglionic blockade with pendiomide (21) could be accounted for by the inability of the sympathetic nervous system to antagonize bronchoconstriction. The slowly adapting bronchial receptors described by Widdicombe (26) can account for this reflex. Kottegod and Mott (23) discuss this possibility with reference to the cat.

Most of the investigations to date have been concerned with the reflex nature of the respiratory action of 5-HT; however, most all investigators state that a residual central mechanism is involved. Stimulation as well as inhibition has been postulated. Marrazzi and Hart (27) first described a transitory inhibition of the ipsilateral transcallosal response of cortical neurons following injection of 5-HT into the carotid artery. Edison (28) was able to show a slower but more prolonged depression of ipsilateral spinal mono- and multisynaptic conduction following large i. v. injections of 5-HT. More recently Koella, *et al.* (29), demonstrated both ipsilateral and contralateral inhibition of optically evoked cortical potentials resulting from carotid artery injection of small doses of 5-HT. Inhibition is more prominent when the carotid sinus is intact but there remains an

inhibition following sinus denervation which they attribute to a direct central effect. They ascribed the reflex and some of the direct inhibition as being mediated by the reticular core which relates directly with the decrease in facilitation of the knee jerk with respiratory inhibition (30).

Inhibition by 5-HT of the gasp reflex elicited by stimulating the carotid body nerve, Fig 7, is an example of the central inhibition of a respiratory reflex. Partial confirmation of this action has been obtained by studying this same reflex in the recipient head of carotid cross-circulated dogs (31). Additional studies have been initiated to define the quantitative as well as qualitative role of 5-HT on central respiratory mechanisms.

## REFERENCES

- (1) Frispmann, V, *Pharmacol Revs*, 6, 125 (1954)
- (2) Comroe, J. H., Jr., Van L., and Street, D. C. and Roucoroni, A., *Am J Physiol*, 1, .
- (3) Page, I. H., *Physiol Rev*, .
- (4) Schneider, J., and Yonkman, 174
- (5) Mott, J. C., and Paintal, A. S., *Brit J Pharmacol*, 8, 238 (1953)
- (6) Paintal, A. S., *Quart J Exptl Physiol*, 40, 89 (1955)
- (7) McCubbin, J. W., Green, J. H., Salmouraglu, G. C. and Page, I. H., *J Pharmacol and Exptl Therap*, 116, 191 (1956)
- (8) MacCanon, D. M., and Horvath, S. M., *Am J Physiol*, 179, 131 (1954)

- (9) Douglas, W. W., and Toh, C. C., *J Physiol*, 120, 311 (1953)
- (10) Ganzel, K. H., *Arch exptl pathol Pharmacol*, 228, 119 (1955)
- (11) Trendelenburg, U., *J Physiol*, 135, 66 (1957)
- (12) Brocklehurst, W., "5-Hydroxytryptamine," Pergamon Press London England, 1958, p. 172
- (13) Feldberg, W., and Sherwood, S. L., *J Physiol*, 123, 148 (1954)
- (14) Feldberg, W., and Smith, A. N., *Brit J Pharmacol*, 9, 31 (1954)
- (15) Neil, G., Redwood, C. R., and Schweitzer, A., *J. Physiol*, 109, 250 (1949)
- (16) Schneider, J. A., and Rinehart, R. K., *Arch intern Pharmacodynamic*, 105, 253 (1956)
- (17) Konzett, H., *Brit J Pharmacol*, 11, 289 (1956)
- (18) Burst, H., Berglund, E., and McGregor, M., *J Clin Invest*, 36, 669 (1957)
- (19) Sparks, C. H., and Tombridge, T. L., *J Thoracic Surg*, 33, 101 (1957)
- (20) Reid, G., and Rand, M., *Nature*, 169, 801 (1952)
- (21) Schneider, J. A., and Yonkman, F. F., *J Pharmacol Exptl Therap*, 111, 84 (1954)
- (22) Heymans, C., and van den Heuvel Heymans, G., *Arch*, 5 (1953)
- (23) C., *Brit J Pharmacol*, 10, .
- (24) Ganzel, K. H., and Kottgoda, S. R., *J Physiol*, 123, 277 (1954)
- (25) Lucco, L., "A Study of the Effects of 5-Hydroxytryptamine (Serotonin) on the Respiratory and Cardiovascular Systems of the Dog," Thesis University of Houston, 1958
- (26) Widdicombe, J. G., *J Physiol*, 123, 55 (1954)
- (27) Marrazzi, A. S., and Hart, E. R., *Science*, 121, 365 (1955)
- (28) Edisen, C., *Arch Neurol Psychiat*, 79, 323 (1958)
- (29) Koella, W. P., Smythies, J. R., and Bull, D. M., *Science*, 129, 1231 (1959)
- (30) Magoun, H., and Rhines, R., *J Neurophysiol*, 9, 165 (1946)
- (31) Kroeger, D. C., Unpublished observation, 1959

# The Quantitative Determination of Free Menthol in Peppermint Oil\*

By MARTIN I. BLAKE†

The free menthol content of peppermint oil is determined by refluxing a sample of oil with chloroacetyl chloride. Hydrogen chloride, a by-product of the reaction, is carried over into a solution of standard silver nitrate with the aid of nitrogen gas. Excess silver nitrate is determined with standard ammonium thiocyanate.

THE OFFICIAL procedure for determining menthol in peppermint oil was developed by Power and Kleber (1) and first appeared in U S P VIII. The method has been frequently criticized and, over the years, numerous modifica-

tions have appeared in the literature. These have been reviewed earlier (2, 3).

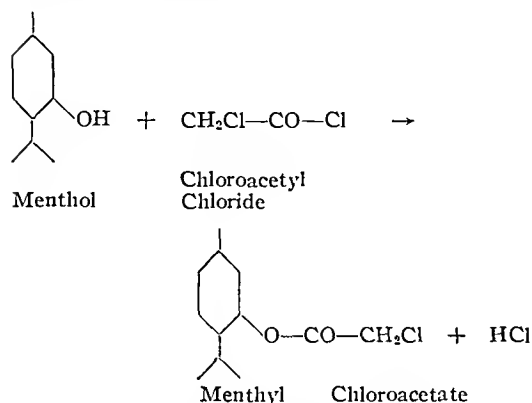
In general, methods proposed for the analysis of menthol take advantage of the reactivity of the hydroxyl group. In the official procedure for peppermint oil (4), the menthol is esterified with acetic anhydride and sodium acetate. Menthol content is evaluated by saponification of the ester with alcoholic potassium hydroxide. Christensen and Pennington (5) determined the menthol content of peppermint oil by acetylation with acetyl chloride. This reagent is also used for determining the hydroxyl number of fatty substances (6).

Methods for determining alcohol constituents in volatile oils are described by Guenther (7). Methods for evaluating alcohols in general, are reviewed by Mehlenbacher (8). These may be

\* Received August 21, 1959 from the School of Pharmacy, North Dakota Agricultural College, Fargo.  
Presented to the Scientific Section, A P H A, Cincinnati meeting August 1959.  
The author graciously acknowledges the financial support of Fritzsche Brothers, Inc., in this project.  
† Present Address: Argonne National Laboratory, Lemont, Ill.

classified as acetylation (acetic anhydride or acetyl chloride), phthalation (phthalic anhydride), and formylation (aceto-formic acid reagent). Alcohols, including menthol, have been determined with lithium aluminum hydride by Lintner, Schleif, and Higuchi (9)

The present report describes a procedure for the estimation of menthol in peppermint oil by acylation with chloroacetyl chloride. The equation for the reaction is:



The hydrogen chloride gas, a by-product of the reaction, is determined by the Volhard method

## EXPERIMENTAL

**Apparatus.**—The arrangement of the apparatus is shown in Fig 1. A standard nitrogen tank was used, and was equipped with a pressure gauge and reduction valve to permit control of the flow of nitrogen

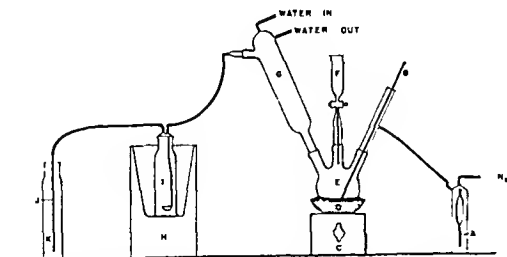


Fig. 1.—Apparatus for determining free menthol in peppermint oil: A, trap; B, thermometer; C, magnetic stirrer; D, heating mantle; E, reaction flask; F, filling tube; G, Friedrichs condenser; H, Dewar flask; I, vapor trap; J, measuring pipet; and K, graduated cylinder.

The outlet of the tank was connected to a trap (A) by tight fitting rubber tubing. The outlet of the trap was joined to a gas inlet of a 500-ml three-necked standard-taper round-bottom flask (E). The reaction flask was fitted with a thermometer (B), a filling tube (F), and a Friedrichs condenser (G). A heating mantle (D) was set beneath the reaction flask and the entire system was mounted over a magnetic stirrer (C). The outlet of the condenser was fitted to a vapor trap (I) having a

fritted disk sealed to the inner tube. The trap was placed in a Dewar flask (H) which was packed with crushed ice during a run. The trap outlet was joined to a small bore 1-ml. measuring pipet (J) by rubber tubing. The pipet was placed in a 50-ml graduated cylinder (K) so that the tip of the pipet extended to about one-half inch from the base of the cylinder. All fittings were tightly secured to minimize the possibility of a gas leak.

**Procedure.**—A sample of pure menthol, 200–300 mg, accurately weighed, was dissolved in 20 ml of toluene, and quantitatively transferred to the reaction flask. One gram of chloroacetyl chloride, dissolved in 20 ml of toluene, was placed in the filling tube. Exactly 25 ml of 0.1 N AgNO<sub>3</sub> solution was transferred by pipet to the 50-ml cylinder. Distilled water was added to the 50-ml. mark. Nitrogen gas was passed through the system at a controlled rate of 120 bubbles per minute as noted in the graduated cylinder. The chloroacetyl chloride solution was added to the reaction flask and the heating unit was turned to "on" position. The solution was stirred gently during the heating period by means of a Teflon covered magnetic bar. The temperature of the reaction was maintained at 101–102°, by means of a rheostat for a period of one hour. After this time, the reaction was stopped and the contents of the 50-ml cylinder were quantitatively transferred to a 250-ml. Erlenmeyer flask. Three milliliters of nitric acid and 3 ml. of nitrobenzene were added, followed by 2 ml. of ferric ammonium sulfate T. S. The excess silver nitrate was titrated with 0.1 N ammonium thiocyanate solution. A blank determination was run in a similar manner, but the menthol sample was omitted and a blank was run after each four determinations.

TABLE I — ANALYSIS FOR FREE MENTHOL

No	Pure Menthol Proposed Method, %	Peppermint Oil		Method of Hamar-nell, et al., %
		Pro-posed Method, %	Official Method, %	
1	101.78	50.93	51.20	50.99
2	100.80	50.90	50.85	50.76
3	100.74	50.26	50.78	50.49
4	100.22	50.01	50.02	50.45
5	99.95	49.76	49.51	50.30
6	99.88	49.46	48.96	50.26
7	99.67	49.10	48.76	50.00
8	98.97	...	...	...
Av	100.25	50.06	50.01	50.46
Av. Dev	0.64	0.55	0.79	0.24

In the analysis of peppermint oil, samples weighing between 400 and 500 mg. (assuming about 50% menthol content) were employed. Samples were conveniently weighed by placing peppermint oil in a half-ounce dropper bottle. The entire system was weighed. Between 16 and 20 drops of oil were added to the reaction flask into which 40 ml of toluene had previously been added. The dropper bottle was again weighed and from the difference in weight the sample weight was readily determined.

Calculations.—Menthol content was calculated from the expression:

$$\frac{\left[ \text{Vol. 0.1 N NH}_4\text{SCN consumed in blank} - \text{Vol. 0.1 N NH}_4\text{SCN consumed in run} \right] \times 15.627 \times 100}{\text{Weight of sample in mg.}} = \% \text{ Menthol}$$

For a comparative study, peppermint oil was assayed by the U. S. P. method (4). Ester content and total menthol were determined. Free menthol was calculated from the results. Comparison was made with the results obtained by the method of Hamarneh, Blake, and Miller (3). All results are reported in Table I.

## DISCUSSION

This paper describes a convenient procedure for the determination of menthol, pure and in peppermint oil. The method is based upon an esterification procedure, the reaction commonly used for the estimation of menthol in peppermint oil. Instead of saponifying the ester, which is the usual procedure, the hydrogen chloride gas, a by-product of the reaction, is passed into a standard silver nitrate solution. This is effected with the aid of dry nitrogen gas which is passed through the system at a controlled rate. Excess silver nitrate is determined with standard ammonium thiocyanate solution.

The reagents employed in this procedure were selected because of their suitability for the conditions of the reaction. The temperature of the reaction was maintained at 101–102°, which was sufficiently high to insure completion of reaction in a reasonable time period, yet low enough that other products would not distil over. Since chloroacetyl chloride has a boiling point of 105°, whereas acetyl chloride boils at 51–52°, the former was selected as the acylating agent. Increase in boiling point is made at no significant expense to chemical reactivity. Toluene, with a boiling point of 111° served well as the reaction solvent. Menthol has a boiling point of 216°, far above the temperature of the reaction. To be certain that only the hydrogen chloride gas would be carried over, it was necessary to take additional precautions. The Friedrichs condenser with cold water passing through, served as a reflux condenser, permitting only gaseous products to pass through.

In addition, the gases passed through a vapor trap maintained at a low temperature. A series of

blank determinations showed a precision of better than 5 parts per 1,000.

The Volhard method was selected for the determination of the hydrogen chloride because the reaction could be followed conveniently by the nature and amount of the precipitate appearing in the graduated cylinder.

Preliminary investigation showed that the reaction, using sample weights indicated in this report, was complete within the one-hour time limit. On the average it required between four and five hours to conduct two runs and a blank. In routine analysis, a series of determinations may be conveniently effected by flushing the system with nitrogen gas prior to the introduction of a new sample and additional chloroacetyl chloride. However, for more accurate work it is suggested that a clean and dry reaction flask and vapor trap be installed for each run.

The results in Table I indicate that the proposed method gives results comparable to the official method and the chromatographic procedure reported earlier by Hamarneh, Blake, and Miller.

Work is currently in progress in this laboratory to apply this procedure to other alcohol-containing volatile oils.

## REFERENCES

- (1) Power, F. B., and Kleber, C., *Pharm. Rundschau*, 12, 162(1894).
- (2) Hamarneh, S. K., M. S. Thesis, North Dakota Agricultural College, Fargo, 1956.
- (3) Hamarneh, S. K., Blake, M. I., and Miller, C. E., *THIS JOURNAL*, 45, 713(1956).
- (4) "U. S. Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 519.
- (5) Christensen, B. E., and Pennington, L., *Anal. Chem.*, 14, 54(1942).
- (6) Jenkins, G. L., Christian, J. E., and Hager, G. P., "Quantitative Pharmaceutical Chemistry," McGraw-Hill Book Co., New York, N. Y., 1957, p. 285.
- (7) Guenther, E., "The Essential Oils," Vol. 1, D. Van Nostrand Co., New York, N. Y., 1948, p. 271.
- "Organic Analysis," Vol. 1, Interscience, New York, N. Y., 1953, pp. 1–65.
- H., and Higuchi, T., *Anal.*



## Notes

### A Note on an Apparatus for Intracerebral Injections in Conscious Mice\*

By J. VANECEK, V. KREBS†, E. SCHEER‡, and T. BIELEKE‡

RECENTLY intracerebral injections in mice have been successfully introduced as a method for studying the direct actions of drugs on the central nervous system with large groups of animals (1). Haley and McCormick (1) presented a detailed methodical account outlining the precise point and depth of injection, but a great degree of experimental skill was necessary for accuracy. Therefore, we tried to facilitate their technique by the following design (Fig 1). It also helps to inject very small volumes of fluid accurately, without using specially accurate syringes.

A head holder (A) has been made from methyl methacrylate to assist in determining the point of injection. On its top, the midline and the points for injection in both ventricles are marked. The head of the mouse is placed in the holder so that the hind border connects the anterior base of the ears. The points for injection are then 2 mm. from the midline on both sides. This corresponds topographically to the point recommended for intracerebral injection in mice (1). The head is held in this position by a plate (B) with a coil spring (C).

In order to prevent the needle from penetrating into the mouth, the insertion depth is fixed at 2.5 mm. As the amount of injection fluid must not exceed 0.05 ml, a micrometer (D) operates the 1-ml. tuberculin syringe (E). This allows the precise administration of volumes of 0.001 ml.

Manipulation with the tuberculin syringe is facilitated with the micrometric screw (F), the weight is equalized by means of a counterweight (G), the thread of which is passed over a pulley (H). Two rods (I) maintain the same position in relation to the points for injection in the holder. The complete apparatus is attached to a stand (J).

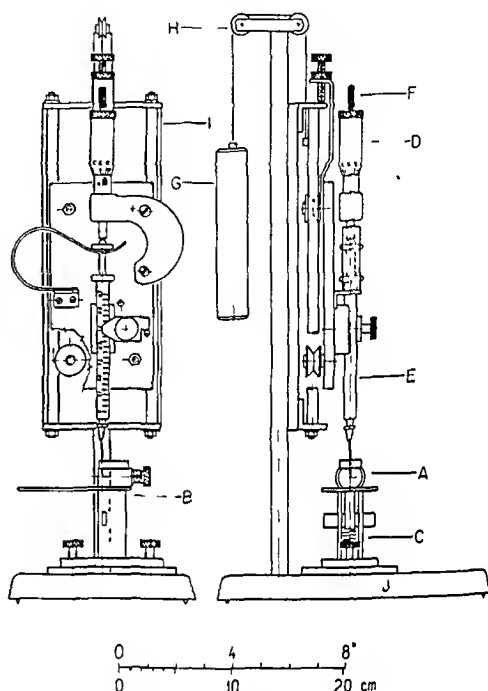


Fig 1—Scale drawing of intracerebral injection apparatus

One hundred mice were injected with methylene blue, and about 85% of the injections were into the ventricle. It is hoped that this design may be of some use with the method of Haley and McCormick.

#### REFERENCE

- (1) Haley, T. J., and McCormick, W. G., *Brit J Pharmacol*, 12, 12(1957)

\* Received November 2, 1959, from the Faculty of Pediatrics, Charles University, Prague, Czechoslovakia.

† Pharmacological Laboratory, Czechoslovakia Academy of Science.

‡ Institute for Corticovisceral Pathology and Therapy, Berlin, Germany.

# A Note on the Correlation of *In Vivo* With *In Vitro* Disintegration Times of Enteric Coated Tablets\*

By JOHN G. WAGNER

IN A PREVIOUS COMMUNICATION (1) the average disintegration time of enteric coated tablets in the intestine of the dog ( $T_{vivo}$ ) was correlated with (a) the average disintegration time of the whole enteric coated tablet in artificial intestinal fluid pH 6.9, ( $T_i$ ) and (b) the average disintegration time of the enteric coating only in the same fluid ( $T_c$ ). The  $T_c$  values were obtained by subtracting the average disintegration time of the corresponding subcoated tablet from the  $T_i$  values. Linear relationships were obtained when  $T_{vivo}$  was plotted against the logarithm of  $T_i$  and when  $T_{vivo}$  was plotted against the logarithm of  $T_c$ . The data used are shown in Table I.

The empirical relationships formerly reported are not supported by theoretical considerations. It has now been found that the above data may be plotted in a different manner to yield linear relationships which do have a theoretical basis. For reactions of order zero to two, the specific rate constant for the reaction is inversely proportional to the time required to reach a certain end point (2). If the reaction order for the disintegration process is from zero

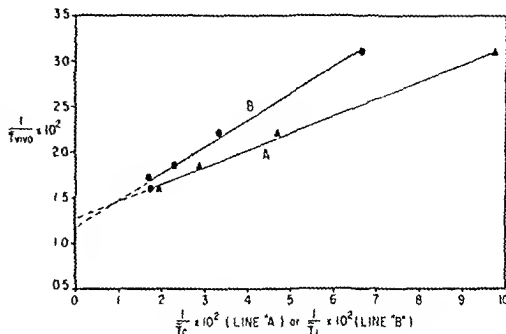


Fig. 1.—Curve A is a plot of the reciprocal of the average intestinal disintegration time in the dog against the reciprocal of the average disintegration time of the enteric coating in artificial intestinal fluid, pH 6.9. Curve B is a plot of the reciprocal of the average intestinal disintegration time in the dog against the reciprocal of the average disintegration time of the whole enteric coated tablet in artificial intestinal fluid, pH 6.9.

TABLE I.—IN VITRO AND IN VIVO DISINTEGRATION TIMES OF ENTERIC COATED TABLETS (TIME IN MINUTES)

Lot	$T_i$	$T_c$	$T_{vivo}$	Type of Enteric Coating
I	15.1	10.3	32.2	Styrene-maleic acid copolymer, dibutyl phthalate, and talc
II	30.1	21.3	45.2	Hydrolyzed resin SC-2, dibutyl phthalate, and talc
III	43.8	35.0	54.0	Cellulose acetate phthalate, propylene glycol, Span 80, and talc
IV	58.2	52.4	62.5	Starch acetate phthalate, propylene glycol, Span 80, and talc
II <sup>a</sup>	59.8	...	58.3	

\* Lot II stored for one month at 47°.

to two, then the specific rate constant will be inversely proportional to the disintegration time. Since the same end point was used both *in vivo* and *in vitro* (essentially complete disintegration of the enteric coated tablets), the reciprocal of the *in vivo* disintegration time may be plotted against the reciprocal of the *in vitro* disintegration time. The reciprocal,  $1/T_{vivo}$ , under the above conditions, will be directly proportional to the specific rate constant for the *in vivo* disintegration process and the reciprocal,  $1/T_i$ , will be directly proportional to the specific rate constant for the *in vitro* disintegration process.

Figure 1 shows a plot of  $1/T_{vivo}$  against  $1/T_c$  (line A) and a plot of  $1/T_{vivo}$  against  $1/T_i$  (line B). The correction coefficient for line A is 0.998 and that for line B is 0.996. Both correlations are highly significant. The equations for the least squares lines are

$$1/T_{vivo} = 0.190 (1/T_c) + 0.0128 \text{ (line A) (Eq. 1)}$$

$$1/T_{vivo} = 0.294 (1/T_i) + 0.0118 \text{ (line B) (Eq. 2)}$$

Assuming the reaction order is between zero and two and is the same *in vivo* as *in vitro*, Eq. 2 indicates that the rate constant for the disintegration process

in the dog's intestine is only approximately one-third of the rate constant for the disintegration process in pH 6.9 intestinal fluid when the whole enteric coated tablet is considered.

Equations 1 and 2 are only valid over the range of disintegration times studied. Equation 2, if utilized outside this range, indicates that as  $T_i$  gets very large, or  $1/T_i$  approaches zero, then  $1/T_{vivo}$  approaches 0.0118, or  $T_{vivo}$  approaches 1/0.0118 or eighty-five minutes. However, it is known from our investigation of lot V in the dog (1) and the work of Chapman, *et al.* (3, 4), in human subjects that tablets which have very large disintegration times may not disintegrate completely, or at all, in the intestines. Hence, one can assume that beyond a certain  $T_i$  value, greater than sixty minutes, the enteric coated tablets will not disintegrate completely in the intestine.

## REFERENCES

- (1) Wagner, J. G., Veldkamp, W., and Long, S., *THIS JOURNAL*, 47, 681 (1958).
- (2) Noddings, C. R., *Chem. Processing*, November, 63 (1953).
- (3) Chapman, D. G., Crisifo, R., and Campbell, J. A., *THIS JOURNAL*, 43, 297 (1954).
- (4) Chapman, D. G., Crisifo, R., and Campbell, J. A., *ibid.*, 45, 374 (1956).

\* Received August 13, 1959, from the Upjohn Co., Kalamazoo, Mich.

# A Note on the Preparation of Saturated Solutions at Constant Temperatures\*

By J. A. WOOD, L. WAIT RISING, AND NATHAN A. HALL

An easily constructed apparatus for the uniform agitation of solute-solvent combinations is described. It can be used effectively in many laboratory mixing problems.

IN CONNECTION with our studies of the solubilization of certain chemicals by surfactants, the construction of an apparatus for uniform agitation of the solute-solvent combination became necessary. By a relatively simple adaptation of materials common to most pharmaceutical research or control laboratories, we have been able to fashion a suitable instrument, the detailed construction of which may be of interest to others faced with a similar problem.

The arrangement, illustrated in Fig. 1a and the photograph, Fig. 1b, makes use of the Vanderkamp tablet disintegration tester (Stoll-Gershberg Type)<sup>1</sup> (a). The apparatus submits the solute-solvent mixtures to a continuous rocking motion which provides the necessary agitation. The vertical shaft (b) is moved up and down within its supporting column in a stroke of 5.5 cm by a special steel-gear reduction motor and an eccentric cam at a fixed speed of 30 cycles per minute. This speed is suitable for limpid and viscous mixtures.

In the assembled disintegration apparatus a basket-rack assembly is fastened to the end of the supporting rod (d) and thereby hangs from the horizontal arm (c). The basket-rack assembly is removed and its supporting rod (d) is connected to an aluminum rod (f),  $\frac{3}{8}$  inch diameter, by means of a piece of thick-walled rubber tubing (e). The terminal  $\frac{3}{8}$  inch of rod (f) is filed flat on two sides and fitted into a slot cut into the end of the tilting arm (h). A hole is drilled through the two pieces of rod and a pin (g) inserted to form a pivot joint.

The tilting arm (h) is a Flexaframe rod (Fisher Scientific Co., Catalog No. 14-666-10) rigidly connected to another Flexaframe rod whose end is visible at point (j) by a Flexaframe connector (i) (Fisher Scientific Co., Catalog No. 14-666-20). Fastened to the rod visible at (j) is a wooden platform (l),  $\frac{1}{2} \times 3 \times 6$  inches, upon which is mounted the solution bottles. The wooden platform is fastened to its mounting rod (j) by means of two aluminum straps placed over the rod and screwed to the wood. The straps used are those which an electrician uses to strap conduit to rafters or walls. The solution bottles (m), only one visible in drawing, are mounted on the wooden table by means of two elastic tapes (n). These secure the bottles but permit easy mounting and removal.

The mounting rod visible at (j) is supported at each end by Flexaframe connectors (k), only one visible in drawing, which are in turn fastened to two supporting rods (o), only one visible in drawing. The lock-screw is removed from connectors (k) so that the rod at (j) is free to rotate. The two

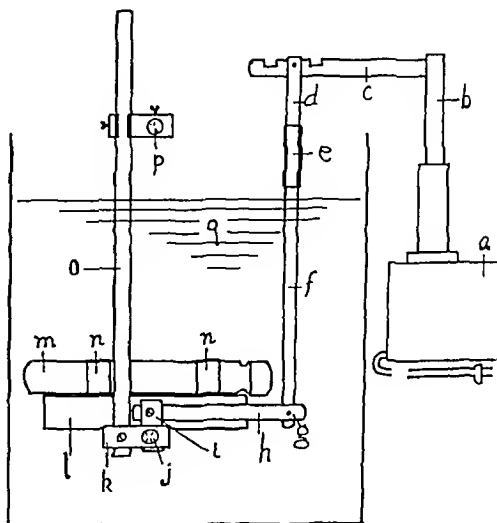


Fig. 1a—Apparatus for preparing saturated solutions

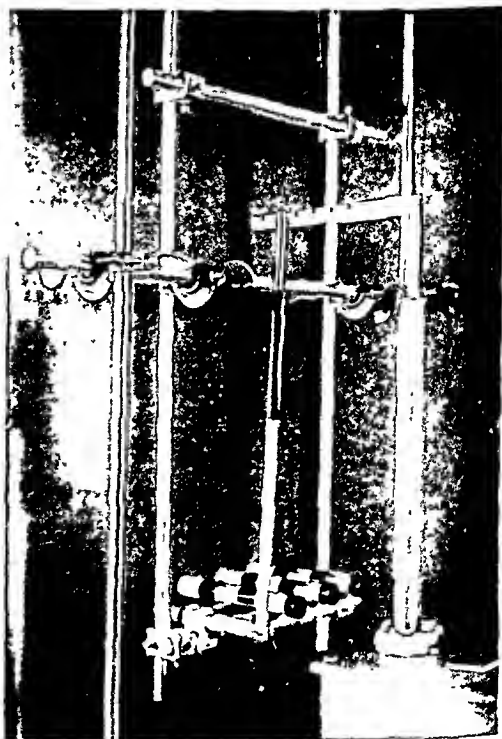


Fig. 1b—Apparatus for preparing saturated solutions.

\* Received August 17, 1959, from the University of Washington, College of Pharmacy, Seattle 5.

<sup>1</sup> Purchased from Parlam Corporation, 340 Canal St., New York 13, N. Y.

support rods (*o*) are maintained at a fixed distance apart by a cross-member rigidly connected near the top of the rods (*p*). The cross member visible at (*p*) is in turn clamped to mounting rods secured to the water bath. This arrangement of supports and connectors serves as a mounting device to suspend the solution bottles in a constant-temperature water bath and as bearings for the operation of the rod supporting the bottles. The water (*q*) provides adequate lubrication for the rod ends (*j*) in the connector bearings. The effective length of the tilting arm (*h*) is 6.3 cm., and it is operated by a

vertical stroke of 5.5 cm., see above; therefore, the angle of tilt through which the solution bottles move is about 50°, sufficient to provide good agitation.

When agitation has continued sufficiently long to saturate the solvent with solute the rod (*d*) is lifted off the supporting arm (*c*) and may be secured by a suitably mounted hook in such a position that the solution bottles are in an upright position. The excess solute is allowed to settle or may be centrifuged to produce a clear supernatant liquid for analysis.

---

## Book Notices

---

*Treatise on Analytical Chemistry.* Part 1: Theory and Practice. Vol. I. Edited by I. M. KOLTHOFF and PHILIP J. ELVING. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. xxvi + 809 pp. 16 x 23.5 cm. Price single \$17.50, subscription, \$15.

This is the first volume of the first of three parts of a comprehensive and systematic treatise on all aspects of classical and modern analytical chemistry, covering the scientific and instrumental fundamentals of analytical methods by critical selection and interpretation of methods and procedures for inorganic and organic compounds. Methods for investigation and evaluation of the properties and composition of commercial products will be included. The first four chapters (Section A) of volume 1 take up "Analytical chemistry: its objectives, functions, and limitations." Section B, chapters 5-19, covers "Application of chemical principles." Each chapter is a contribution by an authoritative worker in the field discussed. References are given at the end of each chapter. Volume 1 does not contain an index but a detailed table of contents is provided. The Treatise is not intended to replace the great number of existing and exhaustive monographs on specific subjects, but rather to serve as an introduction and guide to the efficient utilization of specialized monographs. Volume 1, part 1 is a good beginning.

*Husa's Pharmaceutical Dispensing.* Edited by ERIC W. MARTIN. Mack Publishing Co., 20th and Northampton Sts., Easton, Pa., 1959. ix + 729 pp. 18 x 25 cm. Price \$12.

This textbook has been completely rewritten, with twenty-six authors contributing different chapters. Its style and content are as up-to-date as possible. Particularly interesting is the treatment of "Incompatibilities" in three chapters covering 231 pages. A section on compatibilities and incompatibilities of certain manufactured products is included. The book is directed not only toward helping the individual who compounds prescriptions, but also to the future pharmacists' knowledge about commercial compounding practices as they affect prescription writing by the physician.

*Curare and Curare-Like Agents.* Edited by D. BOVET, F. BOVET-NITTI, and G. B. MARINIBETTÒLO. Elsevier Publishing Co., Amsterdam, 1959. Distributors for the United States, D. Van Nostrand Co., 120 Alexander St., Princeton, N. J. xi + 478 pp. 16.5 x 24 cm. Price \$15.75.

This book is a comprehensive monograph on curare and curare-like agents in which authoritative contributors have written on subjects which are grouped under the following main chapter headings: Ethnographic problems connected with the preparation and use of curare by South American Indians, South American Loganiaceae and menispermaceae as a source of curarizing alkaloids, The Chemistry of naturally occurring curarizing alkaloids, Pharmacological and physiological aspects of curare and curare-like drugs, and Clinical applications of curarizing agents. References are given at the end of each section. A subject index is appended.

*Pain and Itch: Nervous Mechanisms* By Ciba Foundation Study Group No 1. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. vii + 120 pp. 12 x 18.5 cm.

A compilation of the papers and discussions at the symposium of the Ciba Foundation study group No. 1, March 10, 1959. References and an index are appended.

*Analytical Chemistry of Titanium Metals and Compounds.* Chemical Analysis. Vol. 9. By MAURICE CODELL. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1959. xiii + 378 pp. 15 x 23 cm. Price \$12

This is the ninth volume of a series of monographs on analytical chemistry and its applications edited by P. J. Elving and I. M. Kolthoff. The author of this monograph, M. Codell, has gathered information from reliable authorities in the special field covered. The book is designed to provide analytical chemists with the information required to analyze any titanium-containing material. All references as well as author and subject indexes are appended.

*Industrial Gums.* Polysaccharides and Their Derivatives. Edited by ROY L. WHISTLER and JAMES N. BEMILLER. Academic Press, 111 Fifth Ave., New York 3, N. Y., 1959. xi + 766 pp. 15 x 23 cm. Price \$25.

This book presents practical information on industrial gums for the use of researchers, chemical engineers, and technical sales personnel. Discussions cover factors influencing gum costs and applications and the individual gums from agar and gum arabic, through the dextrans and bean and seed gums as well as the cellulose derivatives, to tamarind and ti. A good index is appended.

*Clark's Applied Pharmacology.* 9th ed. By ANDREW WILSON and H. O. SCHILD. Medical Book Dept., Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. xii + 750 pp. 14.5 x 22 cm. Price \$10.

A revised edition with new chapters on the pharmacology of tuberculosis and on psycho-pharmacology, the ninth edition of this textbook correlates the science of pharmacology with the art of therapeutics; focusing on the problems of clinical medicine and the action of drugs upon the functions of the body. The book is designed to stimulate a critical approach to the problems in the complex field of drug therapy.

*Atlas of Human Anatomy.* 5th ed. By FRANZ FROHSE, MAX BRÖDEL, and LEON SCHLOSSBERG. Barnes & Noble, Inc., 105 Fifth Ave., New York 3, N. Y., 1959. x + 180 pp. 13.5 x 21 cm. Price, Paperbound \$2.95, Cloth \$4.50.

This pocket size edition of the Atlas includes page size color reproductions of the Frohse-Brödel wall charts. Also Schlossberg's supplementary charts and an explanatory text. Other color charts (endocrine system) and line drawings are included. An index is appended.

*Methoden der organischen Chemie (Houben-Weyl).* Band 1/2, Allgemeine Laboratoriumspraxis II. Georg Thieme Verlag, Herdweg 63, Stuttgart, N. Germany, 1959. xlv + 1017 pp. 17 x 25.5 cm. Price DM 176.40.

The latest volume of this comprehensive treatise (in German) of the methods and procedures in organic chemistry is the second part of the portion devoted to general laboratory practice. The volumes in this treatise have not appeared consecutively. The publication notice of Band XI/2 on nitrogen compounds, the immediately preceding volume, appeared in THIS JOURNAL, 48, 363(1959). The first part of the present volume deals with methods of comminution and division of materials. The major portion (573 pages) is devoted to general laboratory procedures: from "measurement of liquids by gravity and pump methods" to "heat and cold; measurement, registering, and regulating temperatures." All the sections are liberally illustrated with photographs and diagrams of the apparatus mentioned. The third portion of the book discusses accessory aids in laboratory technique. A final section covers accident prevention in the laboratory, and concludes with a discussion on carcinogenic substances. Author and subject indexes are included.

*A History of the American Dental Association.* By ROBERT W. MCCLUGGAGE. American Dental Association. 222 East Superior St., Chicago 11, Ill., 1959. 520 pp. 16.5 x 23.5 cm. Price \$8, special red leather binding \$12.50.

The stated aim of this book is not to present the history of the American Dental Association as a series of dates, marshalled in chronology and illustrated by faded photographs of bearded men, but rather to present its history against the social and professional background of the times. The story is presented in proper perspective, since the personalities, crises, failures, and achievements which are described integrate this study of dentistry in general, and its professional society in particular, with the overall historical period. The pertinent facts are intertwined with an unusual insight into the various patterns of dental education, research, and practice over the years, which makes reading of the book flow quite easily and naturally.

It is immediately apparent that, in spite of its 520 pages, as much condensation as possible has been achieved. The result is a rapidly moving book with all the necessary facts described as briefly as possible. The historical significance, however, does not suffer by this, but in fact is further enhanced by the excellent documentation with references and explanatory notes grouped by chapters and collected at the end of the book.

While the prime purpose of this publication is to present a history of the ADA, a much broader result has been achieved. The book might equally be regarded as a history of American dentistry—its evaluation, professionalism, and heritage.

*Précis de Chimie Biologique.* Vol. 1. By J. E. COURTOIS and R. PERLÈS. Masson et Cie., 120, boulevard Saint-Germain, Paris, 6<sup>e</sup>, France, 1959. viii + 453 pp. 16 x 21 cm. Price 3,400 fr.

This book (in French) is a concise textbook on biochemistry. It is one of a series of textbooks designed particularly for pharmacy students. The series is appearing under the general title "Collection de Précis de Pharmacie," edited by M.-M. Janot.

*Précis de Pharmacodynamie.* By G. VALETTE. Masson et Cie., 120, boulevard Saint-Germain Paris, 6<sup>e</sup>, France, 1959. viii + 519 pp. 16 x 21 cm. Price 4,200 fr.

This is the third book (in French) in the series "Collection de Précis de Pharmacie," edited by M.-M. Janot for use as textbooks for students of pharmacy and related sciences. It follows the style adopted for this series in that it is a concise presentation of the action of medicaments, or more generally, chemical substances on the living organism.

*Soil, Grass and Cancer.* By ANDRÉ VOISIN. Philosophical Library, Inc., 15 E. 40th St., New York 16, N. Y., 1959. xvii + 302 pp. 13.5 x 21.5 cm. Price \$15.

This book presents the author's views on the relation between the manipulation of the soil and its products and human nutrition. Voisin holds that cancer appears less frequently in dwellers on calcareous areas of different types of soils.

JOURNAL OF THE  
AMERICAN PHARMACEUTICAL  
ASSOCIATION

VOLUME 49

APRIL 1960

NUMBER 4

## An Evaluation of Certain Hypotensive Agents III\*

## Tetrahydroisoquinoline and Tetrahydroquinoline Derivatives

By R. K. BICKERTON†, M. L. JACQUART, W. J. KINNARD, Jr., J. A. BIANCULLI,  
and J. P. BUCKLEY

The hypotensive activity of a series of diquaternarized tetrahydroisoquinoline and tetrahydroquinoline derivatives was investigated in the anesthetized rat. The compounds were evaluated according to the mean duration of action of the dose of the compound producing a mean drop in blood pressure of approximately 50 per cent. Several of the compounds were further evaluated in normotensive dogs. The most active compounds were 1-(1,2,3,4-tetrahydroisoquinolino)-5-dimethylamino-3-hydroxy-3-ethinyl pentane dimethobromide (JB-5037), N-(3-dimethylamino-propyl)-1,2,3,4-tetrahydroisoquinoline dimethobromide (JB-621, IN-243) and 1-(1,2,3,4-tetrahydroisoquinolino)-5-dimethylamino-2-pentyne dimethiodide (JB-679).

BURN AND DALE (1) first described the interruption of ganglionic transmission by tetraethylammonium ion in 1914. Acheson, Moe, and Pereira (2, 3) later described the relatively pure ganglionic blocking properties of tetraethylammonium bromide. Since this time both quaternary and bis-quaternary ammonium compounds have been extensively investigated in search of a compound suitable for use in the treatment of hypertensive vascular disease. O'Dell, Luna, and Napoli (4) reported the hypotensive activity of some bis-quaternary ammonium compounds and found that N-(3-dimethylaminopropyl)-1, 2, 3, 4-tetrahydroisoquinoline dimethobromide possessed the most potent hypotensive activity of a series of tetrahydroisoquinoline derivatives when investigated in dogs. Biel and Di Pierro (5) had previously reported the synthesis and relative hypotensive activity of some quaternary salts of N-(4-amino-

2-butynyl)-1, 2, 3, 4-tetrahydroisoquinoline derivatives. This present report deals principally with the evaluation of the hypotensive activity in rats and dogs of certain diquaternarized tetrahydroisoquinoline and tetrahydroquinoline derivatives (Fig. 1) which have been synthesized by Biel and his co-workers.

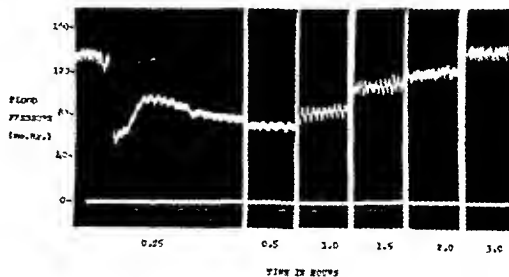


Fig. 1.—The effects of 2.5 mg./Kg. of JB-621 on the blood pressure of a normotensive dog.

## EXPERIMENTAL

**Hypotensive Activity in Normotensive Rats.**—The compounds were screened and evaluated for their hypotensive activity in anesthetized normotensive rats using a modification of a method described in a previous communication from this lab-

\* Received August 21, 1959, from the University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.

† George A. Kelly, Sr., Fellow.

This investigation was supported in part by a research grant from Lakeside Laboratories, Milwaukee, Wis.

H. L. ...  
Pres ...  
meeting, August 1959.

oratory (6). Albino Wistar rats weighing approximately 150 Gm. were anesthetized by an intraperitoneal injection of 1.2 Gm./Kg. of urethan and secured to an animal operating board. The left common carotid artery was cannulated. Normal saline solution was used in the system in place of the 7.5% sodium citrate solution. A minimum of thirty minutes was allowed to elapse prior to drug administration to enable the blood pressure to stabilize. All the compounds were dissolved, just before use, in distilled water prior to being administered into a femoral vein. The initial dose of each compound was 1 mg./Kg., and subsequent dosage was adjusted according to the results obtained in order to determine the dose of a particular compound that would produce a drop in blood pressure of approximately 50%. Dose-responses were plotted on semilog paper and the dose producing a 50% drop in blood pressure was estimated. In some instances it was necessary to utilize a dose of a compound which induced a milder depressor effect because of the obvious signs of toxicity or occasional lethal effect at the higher dose level. The approximate  $ED_{50}$  was then administered to a minimum of four rats (two of each sex), and the mean per cent drop in arterial pressure and the mean time for the blood pressure to return to predrug levels determined. The number of animals used in the evaluation of each compound varied according to the reproducibility of results. Chlorisondamine and pentolinium were used as the control compounds.

**Hypotensive Activity in Normotensive Dogs.**—Certain of the compounds were further evaluated in normotensive dogs to study the possibility of species variation. Mongrel dogs of both sexes were anesthetized with pentobarbital sodium, 35 mg./Kg., I. V. Direct blood pressure recordings were obtained from either the right femoral artery or one of the carotid arteries. Freshly prepared solutions of the compounds were administered via one of the femoral veins.

## RESULTS

**Hypotensive Activities in Normotensive Rats.**—The hypotensive activity of the experimental compounds in rats is summarized in Table I. The compounds were evaluated on the basis of the duration of action of the respective effective doses and not on a dose-dose relationship. The rating used was as follows: +, (relatively poor hypotensive activity), 0–40 minutes duration; ++, (good hypotensive activity), 41–80 minutes; +++, (very good hypotensive activity), 81–120 minutes; ++++, (excellent hypotensive activity), 121+ minutes.

All the experimental compounds showed some degree of hypotensive activity. The most active compounds were JB-5037, 1-(1,2,3,4-tetrahydroisoquinolino)-5-dimethylamino-3-hydroxy-3-ethynyl pentane dimethobromide, JB-621, N-(3-dimethylaminopropyl)-1,2,3,4-tetrahydroisoquinoline dimethobromide, and JB-679, 1-(1,2,3,4-tetrahydroisoquinolino)-5-dimethylamino-2-pentynedimethiodide. The remaining experimental compounds were relatively mild hypotensive agents in comparison to the control compounds investigated.

**Hypotensive Activity in Normotensive Dogs.**—Compounds JB-604, JB-605, JB-610, JB-611, JB-621, JB-633, JB-637, JB-638, JB-679, and JB-5037

were further investigated in normotensive dogs. The results are summarized in Table II. None of the compounds was as active as the esters of piperidine carboxylic acid previously reported by this laboratory (6). Intravenous administration of 0.75 mg./Kg. and 2.5 mg./Kg. of JB-621 to a dog produced a biphasic hypotensive response comparable to that reported by O'Dell (Fig. 1).

All of the compounds produced biphasic drops in pressure with the secondary depressor effect occurring more slowly and usually to a somewhat lesser extent than the initial fall in blood pressure.

## DISCUSSION

In group one, see Fig. 2, the butynyl derivatives of a series of diquaternarized tetrahydroisoquinolines were relatively weak hypotensive agents. However the saturated propyl derivative N-(3-dimethylaminopropyl)-1,2,3,4-tetrahydroisoquinoline dimethobromide (JB-621, IN-243 Irwin, Neisler and Co.) was very active, having a mean duration of action of one hundred and two minutes. The tetrahydroisoquinoline derivative containing an acetylenic branch in the side chain (JB-5037), appeared to be one of the most active compounds investigated. Within group one, acetylenic derivatives, replacement of the terminal alkyl substituted ammonium nitrogen (JB-610) with a heterocyclic structure such as piperidino (JB-620) or pyrrolidino (JB-618) increased the depressor effect but decreased the duration of action of equal doses. Increasing chain length from four carbons (JB-610) to five carbons (JB-679) increased the hypotensive activity four times. It should be noted here that 7.5 mg./Kg. of JB-679 was selected as the rat-evaluating dose although 10 mg./Kg. more closely produced the 50 per cent drops in mean pressure. However four out of six animals died at this dose level (10 mg./Kg.)

In group two, see Fig. 3, similar structure-activity relationships were observed. Replacement of ter-

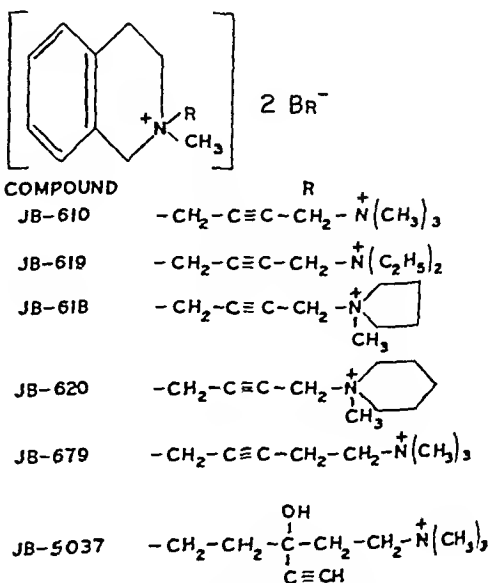


Fig. 2.—Structures of group one.

TABLE I.—THE EVALUATION OF TETRAHYDROISOQUINOLINE AND TETRAHYDROQUINOLINE DERIVATIVES ON THE BLOOD PRESSURE OF NORMOTENSIVE RATS

Compound JB	Dose, mg /Kg	No of Animals	Mean % Drop		Mean Time to Return to Predrug Levels		Activity
			Min	± S D	Min	± S D	
610	20 0	4	62 6	± 22 3	52 0	± 35 7	++
618	5 0	4	44 3	± 8 7	17 2	± 10 5	+
619	5 0	4	49 9	± 12 1	13 0	± 6 7	+
620	5 0	5	40 4	± 10 6	5 1	± 2 3	+
679	7 5	6	34 7	± 7 1	210 5	± 110 1	++++
5037	5 0	12	43 7	± 9 27	139 4	± 65 9	++++
604	20 0	4	47 4	± 19 1	40 6	± 24 0	++
605	20 0	4	48 4	± 6 2	63 0	± 2 1	++
636	10 0	5	51 5	± 11 9	7 0	± 3 8	+
637	10 0	5	50 4	± 3 5	70 2	± 24 5	++
638	25 0	4	48 5	± 1 9	54 5	± 21 7	++
617	5 0	4	42 3	± 8 8	27 5	± 9 9	+
611	5 0	4	38 4	± 5 9	13 1	± 9 7	+
629	10 0	4	48 5	± 8 2	45 25	± 13 3	++
633	10 0	8	44 9	± 5 6	10 6	± 7 1	+
621	5 0	10	57 08	± 15 85	102 66	± 46 6	+++
655	10 0	4	38 2	± 4 4	3 9	± 1 2	+
Pentolinum	1 0	11	48 7	± 18 6	118 5	± 40 3	+++
Chlorisondamine	0 5	14	44 6	± 13 8	141 2	± 51 6	++++

TABLE II —THE EFFECTS OF CERTAIN HYPOTENSIVE AGENTS ON THE BLOOD PRESSURE OF NORMOTENSIVE DOGS

Com- pound JB	Dose, mg /Kg	Mean Drop in Blood Pressure, %	Time to Return to Original Levels, min
604	10	71 6	78
605	10	79 4	113
605	20	49 2	390
610	10	47 3	758+
610	5	54 0	207
633	5	31 4	28
633	30	71 7	198 (died)
637	5	37 9	169
637	10	39 0	121
638	10	50 6	188
638	15	36 8	206
621	5	46 0	327
621	2 5	48 0	170
621	0 75	21 0	80
5037	10	28 0	463
5037	5	27 0	670
5037	2 5	24 0	420
5037	2 5	46 0	247

imal methyl groups (JB-604) with ethyl groups (JB-36) increased the hypotensive depressor effect but creased the duration of action from respective oses The position of the extra nitrogen forming a ydrazine analog appeared critical only when hetero- yche ammonium structures were the terminal roup In this case increased activity was observed hen one of the hydrazine nitrogens was linked to he terminal ammonium nitrogen (JB-637) rather han the tetrahydroisoquinoline nitrogen (JB-638) n group three (Fig 4), tetrahydroquinoline deriva- ves, increased duration of hypotensive activity esulted when the alkyl substituted terminal am- onium nitrogen was replaced with a pyrrolidino adical Depressor responses to equal doses re- mained unchanged

Among the compounds in groups two and three, JB 604, the tetrahydroisoquinoline isomer of JB- 611, required a larger dose to produce a 50% drop in blood pressure but at the same time induced a longer

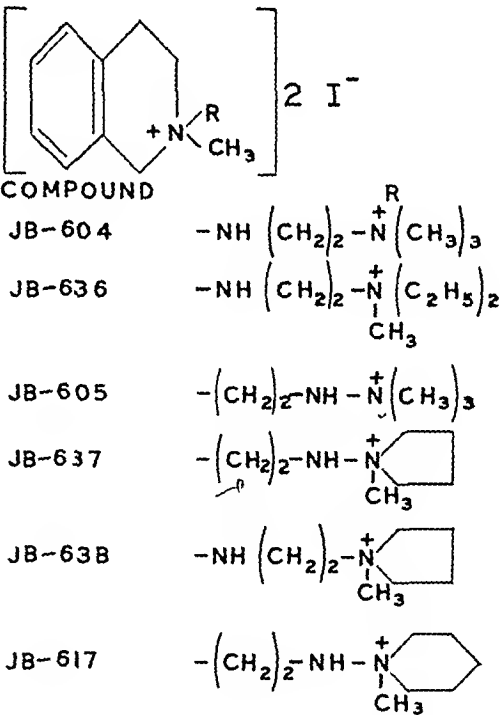


Fig 3 —Structures of group two.

duration of action JB-633 and JB-636, also structural isomers, were qualitatively the same JB-629 and JB-638, the remaining structural isomers, appeared similar in activity excepting that JB-629 was more active on a dose-dose basis

JB-655 (Fig 5), the only tetrahydroisoquinoline carboxylate dimethobromide of the series was also the least active compound JB-621 (IN-243) (Fig. 6) which contains a saturated side chain and an alkyl substituted terminal ammonium nitrogen was one of the most active compounds investigated



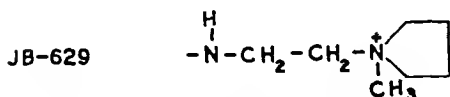
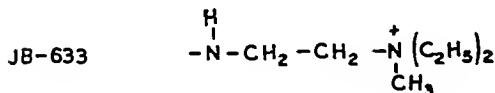
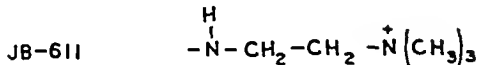
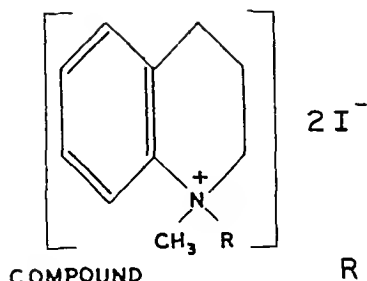


Fig 4.—Structures of group three

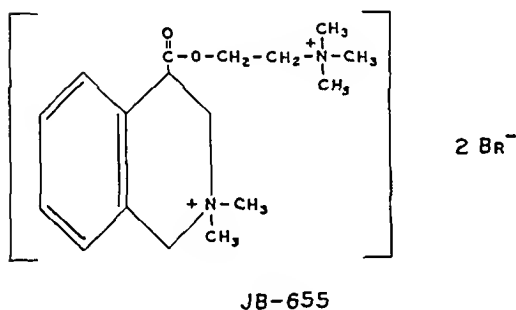


Fig. 5 —Structure of Compound JB-655.

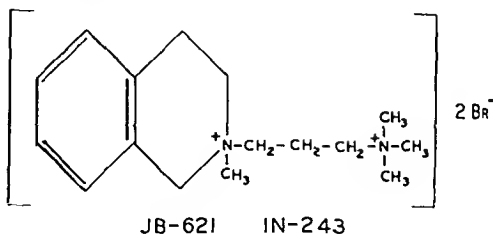


Fig 6 —Structure of Compound JB-621.

## SUMMARY

1 The hypotensive activity of a series of tetrahydroisoquinoline and tetrahydroquinoline derivatives was investigated and evaluated

2 The 2-butynyl derivatives of tetrahydroisoquinoline were relatively weak hypotensive agents as were the diquaternarized hydrazine analogs.

3 JB - 679, 1 - (1,2,3,4 - tetrahydroisoquinolino) - 5 - dimethylamino - 2 - pentyne dimethiodide, JB - 5037, 1 - (1,2,3,4 - tetrahydroisoquinolino) - 5 - dimethylamino - 3 - hydroxy-3-ethynyl pentane dimethobromide, and JB - 621, N - (3 - dimethylamino - propyl)-1,2,3,4 - tetrahydroisoquinoline dimethobromide were the most active compounds in the current series.

## REFERENCES

- (1) Burn, J. H., and Dale, H. H., *J Pharmacol Exptl. Therap.*, **5**, 417(1914)
- (2) Acheson, G. H., and Pereira, S. A. *ibid*, **87**, 273 (1946)
- (3) Acheson, G. H., and Moe, G. K., *ibid*, **87**, 220(1946)
- (4) O'Dell, T. B., Luna, C., and Napoli, M. D., *ibid*, **114**, 317(1955)
- (5) Biel, J. H., and Di Pierro, F., *J Am Chem Soc*, **80**, 4609(1958)
- (6) Hudak, W. J., Buckley, J. P., Schalit, I. M., and Reif, E. C., *THIS JOURNAL*, **46**, 595(1957)

# The Alkaloids of *Argemone munita* subsp. *rotundata*\*

By LEMONT B. KIER† and TAITO O. SOINE

Continuing studies in these laboratories with respect to the alkaloidal content of *Argemone* species have led the authors to examine *Argemone munita* subsp. *rotundata* obtained from the vicinity of Nephi, Utah. The usual isolation techniques have led to a crystalline base (m. p. 245–245.5°), provisionally named *rotundine*, as the principal alkaloid in the overground portions of the plant. Other alkaloids in the plant material are present in minute amounts and have not been obtained in sufficient quantity for detailed study. Analysis of *rotundine* established the formula,  $C_{19}H_{21}NO_4$ , which has been expanded further by functional group analysis to  $C_{16}H_{10}(OCH_3)_2(OH)_2(NCH_3)$ . Methylation of the phenolic base with diazomethane yielded a base,  $C_{21}H_{23}NO_4$ , identical with argemonine, a base previously isolated from *Argemone hispida*. Pharmacologic examination of the new base revealed a slight to moderate inhibition of motor activity as the principal action combined with a low toxicity.

**A**RGEMONE MUNITA Dur. and Hilg. subsp. *rotundata* (Rydb.) G. B. Ownb. is a perennial found growing along roads, washes, and denuded land and is regarded as a pioneer species. It has been known by a number of other names including *A. rotundata* Rydb., *A. hispida* Torrey, and *A. Mexicana* Torrey. Henceforth in this paper, the plant will be referred to as *A. munita* subsp. *rotundata*. It is found in Utah, Nevada, and southern California. The plant itself has a stout stem which is densely and strongly bristled. The cauline leaves are bristly, especially on the veins and margins and they are usually round-lobed. The calyx also is bristly with erect or slightly spreading horns. The petals are white and fully 4–6 cm. long. The pod is ovate to narrowly elliptical and very bristly.

A search of the literature reveals that *A. munita* subsp. *rotundata* has not been examined for its alkaloidal content under any of its botanical names. A few other species have been studied, namely *A. Mexicana* (1–6), *A. alba* (7), *A. platyceras* (8) and, *A. hispida* G. (9, 10). The first three species named have contained alkaloids, principally of the cryptopine, berberine, and sanguinarine types. The bases in *A. hispida* G., however on the basis of a preliminary study (10), do not appear to conform to these types. According to Ownbey (11), it is definitely possible that *A. munita* subsp. *rotundata* is the same species that was originally investigated by Soine and Gisvold (9) as *A. hispida* G. The true species, *A. hispida* G., is said to be found in a narrow belt

running down the eastern side of the Rocky Mountains from southeastern Montana, through Colorado, to northern Arizona whereas the species investigated by Soine and Gisvold was collected in the vicinity of Reno, Nevada, where only *A. munita* subsp. *rotundata* is found. It would appear, therefore, that it was impossible for *A. hispida* G. to have been the subject of their investigation and that they actually studied *A. munita* subsp. *rotundata*. Unfortunately, for lack of sufficient material, the authors were unable to study the Nevada-grown *A. munita* subsp. *rotundata* to prove or disprove this fact. Specimens of the Nevada plant, however, have been compared with Utah-grown *A. munita* subsp. *rotundata* and found to be morphologically identical. Inasmuch as a plentiful supply of the Utah-grown plant was available, it was chosen as the material for further study.

The results of this investigation have indicated a difference in the alkaloids found as against those previously isolated from *A. hispida* G. The alkaloids found earlier were nonphenolic argemonine ( $C_{21}H_{23}NO_4$ ) and mono-phenolic *norargemonine* ( $C_{20}H_{23}NO_4$ ), the latter being convertible to the former with diazomethane. The principal new alkaloid, provisionally named *rotundine* ( $C_{19}H_{21}NO_4$ ), isolated in the present study, was diphenolic and, while different from the preceding, also was closely related because it could be methylated to yield argemonine. Therefore, one can assume with assurance that the basic alkaloidal nucleus is the same and it is probable that the originally studied *A. hispida* G. was, indeed *A. munita* subsp. *rotundata* with the alkaloidal differences being accounted for by soil and climatic differences.<sup>1</sup>

Isolation of the alkaloids from *A. munita*

\* Received August 21, 1959, from the College of Pharmacy, University of Minnesota, Minneapolis.

† Samuel W. Melendy Fellow, 1957. Present address: College of Pharmacy, University of Florida, Gainesville.

The authors are indebted to Prof. Gerald B. Ownbey, Department of Botany, University of Minnesota, for his advice, counsel, and professional identification of all plant materials. Because he has recently reclassified the entire *Argemone* genus (11) and is probably the country's leading expert on the genus, the authors felt extremely fortunate in being able to obtain his services.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

<sup>1</sup> Batra and Soine (12) have recently examined a sample of true *A. hispida* G. collected in Colorado and have been able to isolate both argemonine and norargemonine as well as to note the presence of other unidentified alkaloids.

subsp. *rotundata* was carried out by the usual method of extracting the dried and powdered over-ground portions of the plant material with alcohol,<sup>2</sup> followed by removal of the solvent and transfer of the alkaloidal bases to a dilute hydrochloric acid solution. This solution of alkaloidal hydrochlorides was then alkalinized with sodium carbonate and extracted with an immiscible solvent to obtain a crude total alkaloidal fraction. These were placed into an acidic solution again and separated into phenolic and nonphenolic fractions by making use of sodium hydroxide as the alkalinizing agent to hold the phenolic bases in the aqueous phase as phenolates while the nonphenolic bases were extracted with an immiscible solvent. The phenolic bases were finally obtained from the alkaline solution by carbonation or by reacidification and sodium carbonate alkalization followed by immiscible solvent extraction. Attempted purification of the nonphenolic fraction by recrystallization and chromatography indicated the presence of at least two components but the amounts were so small that further work on this fraction was discontinued. Insofar as we were able to determine, argemone did not appear to be present in the nonphenolic fraction. The phenolic fraction appeared to represent the bulk of the alkaloids present in the plant. This fraction was repeatedly recrystallized and appeared to be composed of a single entity, provisionally named *rotundine*. The homogeneity of *rotundine* was established by paper chromatography. Elemental analysis indicated the presence of carbon, hydrogen, nitrogen, and oxygen. Functional group analysis indicated the presence of two methoxyl groups, one N-methyl group, and two active hydrogens. The presence of two active hydrogens combined with the phenolic character of the base suggested two phenolic groups. From the elemental analysis and molecular weight determination, the molecular formula,  $C_{19}H_{21}NO_4$ , was assigned. This was extended to  $C_{16}H_{10}-(OCH_3)_2(OH)_2(NCH_3)$  by the functional group analysis. Optical rotation studies showed that rotundine was strongly levorotatory. Infrared spectra showed strong absorption in the hydroxyl region, confirming the phenolic character, but no absorption was observed in the carbonyl region (see Fig. 1). Methylation of rotundine with diazomethane produced a dimethyl ether with alkaloidal character analyzing for the formula,  $C_{21}H_{25}NO_4$ . This dimethyl ether was found to be identical with argemone, previously isolated from *A. hispida* G. (9, 10), by comparison of de-

rivatives as well as by comparison of the infrared spectra of the methiodides (see Fig. 2). Soine and Schermerhorn (10) had previously reported that norargemone, when methylated, gave argemone and, therefore, the relationship between these three alkaloids was established. Confirmation of this was found in the similar ultraviolet spectra of the three compounds (see Fig. 3), all apparently containing the same chromophore. A future communication will deal with structural studies that have been carried out on the three alkaloids.

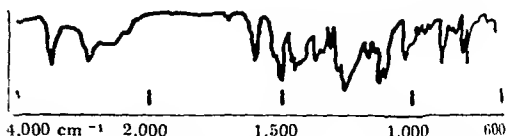


Fig. 1.—Rotundine; phase, potassium bromide.



Fig. 2.—Dimethylrotundine methiodide; phase, potassium bromide.

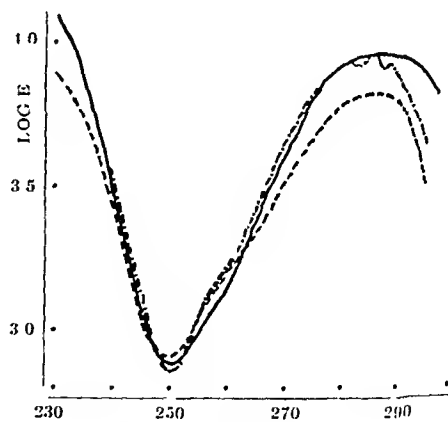


Fig. 3.—Wavelength in mμ.

## EXPERIMENTAL

**Plant Material.**—The above-ground portions of the plant were collected in the late summer of 1954 and 1956 near Nephi, Utah, by Professor Bertrand F. Harrison of the Botany Department of Brigham Young University at Provo, Utah. The plants were identified as *Argemone munila* subsp. *rotundata* by Prof. Gerald B. Ownbey, University of Minnesota, and a voucher specimen was deposited in the University of Minnesota herbarium. The plant material was air-dried and, finally, ground to a 20-mesh fineness for extraction. Preliminary qualitative tests for the presence of alkaloids were positive.

**Extraction.**—Seven kilograms of powdered plant material was macerated for one hour with alcohol,

<sup>2</sup> In this paper "alcohol" will be taken to mean alcohol U. S. P. XV.

placed in a Lloyd extractor, and continuously extracted for ninety hours. The marc showed the absence of alkaloidal bases when tested qualitatively. The alcoholic extract was concentrated *in vacuo* to remove as much alcohol as possible. Water was added periodically to the extract to replace the alcohol that was removed and this process was continued until the alcohol was completely removed. The aqueous mixture was acidified sufficiently to make it approximately 5% with respect to hydrochloric acid. The acidic solution was removed from the dark sludge, which was worked up several times with additional portions of 5% hydrochloric acid until the sludge was free of alkaloid. All of the acidic extractions were combined and rendered alkaline with saturated sodium carbonate solution. The liberated bases were completely extracted from the aqueous solution by extraction in a separatory funnel with many portions of ether. The combined ethereal extracts were concentrated to a convenient volume and then extracted with 5% hydrochloric acid to obtain an acidic solution of the alkaloidal bases. This acidic solution was then rendered strongly alkaline by the use of a 25% sodium hydroxide solution and extracted completely with ether until the ethereal extracts showed no test for alkaloids although the alkaline aqueous phase gave a strong alkaloidal test. The ethereal extracts were combined, washed, and dried for further examination of the nonphenolic alkaloids. The strongly alkaline solution remaining in the separatory funnel was then converted to a mildly alkaline solution by carbonation through the addition of excess solid carbon dioxide, a process which released the phenolic alkaloids as free bases. These were extracted either with ether or dichloromethane, the latter being preferable, until the aqueous phase failed to show an alkaloidal test. The solution of phenolic bases in the immiscible solvent was then washed with water and dried over anhydrous sodium sulfate.

**Nonphenolic Fraction.**—The ethereal solution of nonphenolic bases was distilled under reduced pressure to yield 1.2 Gm. of a dark brown residue, corresponding to a yield of 0.015% based on the dry weight of the crude plant material. The crude alkaloidal residue was very soluble in dilute acid, acetone, and methanol but insoluble in dilute base, water, benzene, and petroleum ether. Attempts to crystallize the crude material were unsuccessful. The material (600 mg.) was then chromatographed on an alumina column (18 Gm.) and eluted with benzene-ether mixtures (from 3:1 to 1:3), and finally with pure ether. As a result of the chromatographic procedure, an almost colorless residue was obtained giving a strong alkaloidal test and weighing 220 mg. The dark brown color remained on the column and apparently was nonalkaloidal because, when washed off with methanol, it failed to give an alkaloidal test. A small portion of the alkaloidal residue from the eluted fraction was dissolved in alcohol and treated with an excess of methyl iodide but failed to yield a methiodide even on refrigeration.<sup>3</sup>

Another small portion of this material was treated with a drop of concentrated sulfuric acid and found to give a lavender-colored solution which quickly turned to orange. The remainder (200 mg.) of the alkaloidal fraction was rechromatographed on aluminum oxide (6 Gm.) with chloroform as the eluant. Two fractions were obtained, the first consisting of approximately 60 mg. and the latter of about 100 mg. Although the first fraction was able to be recrystallized from Skelly B, the amount of material became vanishingly small and the best melting point recorded was 125–134°. The second fraction was in the form of a pink amorphous powder which resisted recrystallization and failed to give a methiodide from an alcoholic solution. Because of the small amounts of material in the nonphenolic fraction, the work on this material was discontinued.

**Phenolic Fraction.**—The ether (or dichloromethane) solution of the phenolic alkaloids was concentrated under reduced pressure to yield a yellowish-white semicrystalline residue representing 0.067% of the crude plant material taken. A portion of this residue was seemingly quite soluble in alcohol whereas a crystalline white residue remained which was considerably more resistant to solution in alcohol. Although a portion of the residue went into solution with alcohol, the majority of the phenolic fraction was in the difficultly soluble residue and it was later found that even the material that went into alcohol so readily was largely of the less soluble type when freed of contaminate. We have not been able to find another alkaloid in this fraction although the conventional techniques of crystallization and chromatography have been employed. The best solvent for recrystallization of the white, crystalline residue was found to be methanol with repeated recrystallizations yielding lustrous, white leaflets, m. p. 245–245.5°.<sup>4</sup>

The crystalline material is soluble in dilute acid, dilute sodium hydroxide, difficultly soluble in methanol, ethanol, and chloroform and insoluble in acetone, water, and dilute sodium carbonate solution. The ascending paper chromatographic technique of Levine and Fischbach (13), as modified for slow moving alkaloids, was used to determine if more than one alkaloid were present. Repeated experiments showed only one alkaloid with an  $R_f$  value of 0.29. The alkaloid appears to be a new one and has provisionally been named "rotundine" after the subspecies of the plant.

**Rotundine.**—A highly purified sample of rotundine was dried *in vacuo* at 100° in an Abderhalden drying pistol employing phosphorus pentoxide as a desiccant for three hours. The sample lost no weight so it was assumed that solvent of crystallization was probably absent. The specific rotation of rotundine was found to be  $[\alpha]_D^{25} = -265.8^\circ$  ( $c = 0.153$ ) in methanol.

**Anal.**<sup>5</sup>—Calcd. for  $C_{12}H_{19}(OCH_3)_2(OH)_2(NCH_3)_2$ : C, 69.73; H, 6.59; N, 4.28; —OCH<sub>3</sub>, 18.96; —NCH<sub>3</sub>, 4.59; Active H, 0.61 (Zerewitinoff). Found: C,

<sup>4</sup> All melting points were determined with the Kofler apparatus.

<sup>3</sup> This behavior differs markedly from that of argemone, which readily forms a methiodide in alcoholic solution even in the presence of other contaminating bases. For this reason, it might be suspected that argemone is absent from this fraction.

<sup>5</sup> The analyses in this work were performed by Clark Microanalytical Laboratories at Urbana, Ill., Weiler and Strauss Microanalytical Laboratory at Oxford, England, and the Microanalytical Laboratory in the Chemistry Department, University of Minnesota.

70.02; H, 6.31; N, 4.30;  $-\text{OCH}_3$ , 19.22;  $-\text{NCH}_3$ , 3.31; Active H, 0.55.

For the above formula, the calculated molecular weight was 327. An ebullioscopic molecular weight determination using methanol as the solvent showed an experimental value of 323. The use of ethyl methyl ketone as a solvent gave a value of 497, indicating the possible combination of the solvent into a molecular complex with the alkaloid [hemiketal(?)].

An alcoholic solution of rotundine imparted an emerald green color when treated with a few drops of 5% ferric chloride solution.

**Methylation of Rotundine.**—Rotundine (0.2 Gm.) was dissolved in approximately 25 ml. of absolute methanol and to this was added a fivefold molar excess of diazomethane in ether maintaining a temperature of not over 5° in the reaction mixture. The solution was allowed to stand at this temperature for two hours and then allowed to come to room temperature slowly. The excess diazomethane and ether were removed with gentle heat and the methanol was removed under an air stream. The amorphous residue was then dissolved in 25 ml. of 5% hydrochloric acid and the acidic solution made strongly alkaline with 25% sodium hydroxide solution. The alkaline solution was then thoroughly extracted with ether to remove the nonphenolic base. The ether extracts were dried over anhydrous sodium sulfate and the ether removed by gentle heating. The residue was recrystallized from a dilute alcoholic solution to finally give prismatic crystals, m. p. 130–135° (with effervescence) (capillary). The crystals were pulverized in a mortar and then dried for four hours at 100° in an Abderhalden drying pistol charged with phosphorus pentoxide. The dried alkaloidal base melted sharply at 151–151.5° (capillary) and a mixed melting point determination with an authentic sample of argemonine failed to depress the melting point.

The methylated rotundine (0.1 Gm.) was converted to the pierate in the usual manner and recrystallized several times from boiling water, m. p. 241–242° (capillary). A mixed melting point in a capillary with an authentic sample of argemonine pierate (9) failed to depress the melting point.

Methylated rotundine (0.1 Gm.) was also converted to the styphnate in the usual manner and recrystallized several times from boiling water, m. p. 247–248° (capillary). A mixed melting point with authentic argemonine styphnate failed to depress the melting point.

Methylated rotundine (0.22 Gm.) was dissolved in 5 ml. of alcohol and treated with 2 ml. of methyl iodide. A crystalline deposit of the methiodide formed within a few minutes. This was removed by filtration and recrystallized from alcohol several times, m. p. 273–274° (decompn.). A mixed melt-

ing point with authentic argemonine methiodide failed to depress the melting point. The infrared spectra of the two methiodides was also compared (see Fig. 2) and found to be identical in all respects.

**Ultraviolet.**—Highly purified samples of rotundine, argemonine, and norargemonine (9) were dissolved in alcohol and suitable dilutions made in order that their characteristic absorption curves in the ultraviolet range could be ascertained. Both the conventional Beckman DU instrument and the automatically recording Cary instrument were utilized, both giving virtually identical curves. All three of the alkaloids showed a minimum at 250  $m\mu$  and the maxima were found to be 288, 287, and 287  $m\mu$ , respectively, for rotundine, norargemonine, and argemonine (see Fig. 3). The curves were essentially identical although argemonine showed well-defined shoulders at 280.2 and 291.2  $m\mu$ .

**Pharmacological Testing.**<sup>6</sup>—The predominant effect in mice is a slight to moderate decrease in motor activity with an oral dose of 500 mg./Kg. On the same mice, death occurred with an oral dose of 2,000 mg./Kg., but even at these large doses no significant biological activity was observed. No change in mean arterial blood pressure of the anesthetized cat was observed after acute intravenous doses of 20 mg./Kg. or 39 mg./Kg. cumulative doses. No changes in the autonomic nervous system were observed. In general, even with the weak central nervous system depressant effect observed in mice, the action of the alkaloid was considered to be so slight that its value as a possible therapeutic agent was considered very unlikely.

## REFERENCES

- (1) Charbonnier, L. J. *Pharm. Chem.*, **7**, 348 (1868).
- (2) Schlotterbeck, J. O., *Proc. Am. Pharm. Mfrs. Assoc.*, **49**, 247 (1901).
- (3) Santos, A. C., and Adkilen, P., *J. Am. Chem. Soc.*, **54**, 2923 (1932).
- (4) Almeida Costa, O. de, *Bol. assoc. brasil. pharm.*, **14**, 489 (1933); through *Chem. Abstr.*, **28**, 1811 (1934).
- (5) Almeida Costa, O. de, *Rev. flora med. Rio de Janeiro*, **1**, 49, 1546 (1949).
- (6) Lazurevskii, C. V., *dy Uzbekskogo Gosudarst. Univ.*, **15**, 182 (1939); through *Chem. Abstr.*, **35**, 4154 (1941).
- (9) Soine, T. O., and Gisvold, O., *THIS JOURNAL*, **33**, 185 (1944).
- (10) Schermerhorn, J. W., and Soine, T. O., *ibid.*, **40**, 19 (1951).
- (11) Ownbey, G. B., *Memoirs of the Torrey Botanical Club*, **21**, 67 (1958).
- (12) Batra, K. V., and Soine, T. O., unpublished study.
- (13) Levine, J., and Fischbach, H., *THIS JOURNAL*, **44**, 43 (1955).

<sup>6</sup> The authors are indebted to the Smith Kline and French Laboratories, Philadelphia, Pa., for testing rotundine for any significant pharmacological effects. We are especially indebted to Dr. Robert F. Raffauf for handling the details of our requests for assistance.

# Preparation and Properties of New Gastric Antacids I\*

## Aluminum Hydroxide-Magnesium Carbonate Dried Gels

By STEWART M. BEEKMAN

The preparation and properties of two new, highly reactive aluminum hydroxide-magnesium carbonate dried gels (AHMC) are described. The rate of reaction with gastric acid is shown to be rapid as well as prolonged in the optimum pH range of 3 to 5. Three methods were used to determine reaction rate including the Reheis and Murch reaction velocity tests as well as a modified procedure of Holbert, Noble, and Grote. An automated apparatus for antacid evaluation is described. Data on thirteen antacid chemicals as well as thirty of the most widely used antacids in liquid and tablet form are presented. The *in vitro* antacid properties of AHMC dried gels compares favorably with reactive liquid aluminum hydroxide gel. The aging characteristics are shown to be excellent.

SINCE ITS INTRODUCTION into therapeutics in 1934 by Einsel, Adams, and Myers (1), aluminous liquid aluminum hydroxide gel, alone or in effective combination with other antacids such as magnesium hydroxide or magnesium silicate, has become the antacid of choice for the medical management of peptic ulcer and gastric hyperacidity. The literature is replete with reports of its successful use in the clinical treatment of these disorders (2-18). *In vitro* antacid evaluation tests carried out by many investigators (19-26) have shown that liquid aluminum hydroxide gel of proved clinical effectiveness yields both prompt and prolonged activity in the optimum pH range of 3 to 5. It also maintains this activity during a long shelf life.

In contrast with the ideal behavior of the liquid product, dried aluminum hydroxide has been shown (24, 27) to be slow in reacting with gastric strength acid, markedly inhibited in that action by pepsin, and to exhibit a diminution in reaction rate on aging.

This paper and others to follow will present *in vitro* antacid evaluation data on several new aluminum hydroxide gels in dry form which compare favorably in their antacid action with liquid aluminum hydroxide gel. The new dried gels have been designed to exhibit (a) promptness of reaction with gastric strength acid, (b) prolonged reactivity in the pH range of 3 to 5 in the presence of pepsin, (c) minimum loss of reactivity on aging, and (d) to be made generally available at moderate cost. The variety of dried gels to be made available will enable the pharmaceutical manufacturer to select a product which best meets his requirement.

This paper will describe two new aluminum hydroxide-magnesium carbonate dried gels (AHMC) which are designated as type F-MA11 and type F-MA12. They differ only in the mole ratio of alumina to magnesia present which is 2:1 and 1.25:1, respectively. When a highly reactive and stable aluminum hydroxide gel is intimately blended with a freshly prepared, low temperature, magnesium carbonate gel and the whole carefully reduced to dry form as by spray drying, the resulting white, free flowing, dense, tasteless powder is found to rehydrate readily and react rapidly with gastric strength acid containing pepsin.

### EXPERIMENTAL

The average composition and physical properties of the two new AHMC dried gels are shown in Table I.

TABLE I.—ANALYSES OF TYPICAL SAMPLES OF AHMC DRIED GEL

	F-MA11	F-MA12
Aluminum oxide ( $\text{Al}_2\text{O}_3$ )	42.0%	37.1%
Magnesium oxide ( $\text{MgO}$ )	8.3%	11.7%
Carbonated ( $\text{CO}_2$ )	20.0%	16.2%
pH 4% aqueous suspension	9.2	9.1
Acid consuming capacity, (ml. 0.1 N HCl per Gm.)	287	286
$\text{Al}_2\text{O}_3$ : $\text{MgO}$ Mole Ratio	2:1	1.25:1
Apparent density (cc./Gm.)	0.44	0.2 or 0.4 <sup>a</sup>

<sup>a</sup> Depending on method of drying.

**X-ray Diffraction Studies.**—X-ray diffraction studies were made using the Debye-Scherrer powder technique with thin-wall glass capillaries 0.30 mm. O. D. and 0.25 mm. I. D. The exposures were made for two hours at 35 mv. and 20 ma. on the X-ray tube. Exposures were made of aluminum hydroxide dried gel, magnesium carbonate gel dried, the new AHMC dried gels, and a dry blend of aluminum hydroxide dried gel with magnesium carbonate powder. Aluminum hydroxide dried gel and AHMC dried gels gave no pattern, indicating that

\* Received September 2, 1959, from the Research Laboratory, Reheis Co., Inc., Berkeley Heights, N. J.  
Presented to the Scientific Section, A. Ph. A. Cincinnati meeting, August 1959.

they are essentially, if not wholly, amorphous. The dry blend gave a faint pattern with lines corresponding to magnesium carbonate. The dry magnesium carbonate gave a very distinct pattern.

**Infrared Spectra.**—Rock salt infrared spectra (2 to 15  $\mu$ ) were made on samples of AHMC dried gel as well as each component dried separately. Nujol as well as hexachlorobutadiene (HCBD) mulls were made. A careful examination of the absorption bands of the two components showed that they were present in the absorption spectra of AHMC with no new bands present in the latter. It was concluded that AHMC dried gels are simple mixtures and not new compounds. The infrared spectrum of magnesium carbonate showed that the material chemically is intermediate between  $MgCO_3$  and  $3 MgCO_3 \cdot Mg(OH)_2 \cdot 3H_2O$ .

**Antacid Activity.**—Four principal *in vitro* methods are used to evaluate antacids in our laboratories as follows: (a) acid consuming capacity, (b) Reheis reaction velocity test, (c) Mutch reaction velocity test, and (d) procedure of Holbert, Noble, and Grote modified.

**Acid Consuming Capacity.**—This measures the volume of 0.1N HCl with which 1 Gm. of sample may react at 37.5° for one hour with an excess of acid present. The excess acid is back titrated to the Toeplers reagent end point (pH 3.5). Since this test does not show rate of change and since it employs an excess of acid throughout, it has been classified as generally unsatisfactory (21, 28–30). Values obtained for various antacid chemicals are shown in Table II.

TABLE II.—ACID CONSUMING CAPACITY OF VARIOUS ANTACIDS

Sample	ml. 0.1 N HCl/Gm. Sample
AHMC type F-MA11	287
AHMC type F-MA12	286
Aluminum dihydroxy aminoacetate	175
Aluminum dihydroxy sodium carbonate	267
Sodium polyhydroxy aluminum monocarbonate hexitol complex	275
Aluminum hydroxide dried gel	267
Sodium bicarbonate	120
Magnesium carbonate	220
Calcium carbonate	210
Magnesium trisilicate	115
Magnesium oxide	495

**Reheis Reaction Velocity Test.**—This test has been used by the author in our laboratories for eighteen years as a relatively quick method of assessing reaction rates of alumina gels. The weight of sample containing the equivalent of 0.5 Gm.  $Al_2O_3$  is added to 100 ml. 0.1N HCl at 37.5° and the whole shaken until the pH rises to about 3.5 (Toeplers reagent). The time is noted and is that required for the sample to react with 34% of the theoretical amount of acid.

A highly reactive alumina gel gives values of ten to twenty seconds. A newly prepared sample of spray dried gel may react in one hundred and twenty seconds and two hundred and forty seconds after three months storage at room temperature. After four years of storage the time may increase to six hundred and sixty seconds. By contrast, samples of AHMC yield values of twenty seconds when new and similar

values after two years aging at room temperature. Typical values for various aluminum-containing antacids are shown in Table III.

TABLE III.—REHEIS REACTION VELOCITY VALUES FOR VARIOUS ALUMINUM ANTACIDS

Sample	Seconds
Aluminum hydroxide gel U. S. P.	15
Aluminum hydroxide dried gel U. S. P.	240
AHMC type F-MA11	22
AHMC type F-MA12	20
Sodium polyhydroxy aluminum monocarbonate hexitol complex	35
Aluminum dihydroxy aminoacetate	29
Aluminum dihydroxy sodium carbonate	15
Aluminum hydroxide dried gel U. S. P.-magnesium carbonate U. S. P. dry blend (4:1)	247

**Mutch Reaction Velocity Test.**—This test which was described by Mutch (31) in 1946 is similar to the Reheis reaction velocity test described above except that it uses 78% of the theoretical amount of acid per weight of alumina. The time is that required for the sample containing the equivalent of 0.10 Gm.  $Al(OH)_3$  to react with 30 ml. 0.1N HCl at 37.5° to the Toeplers reagent end point. Typical values for various aluminum-containing chemicals are shown in Table IV.

TABLE IV.—MUTCH REACTION VELOCITY VALUES FOR VARIOUS ALUMINUM ANTACIDS

Sample	Seconds
Aluminum hydroxide gel U. S. P.	40
Aluminum hydroxide dried gel U. S. P.	1,080
AHMC type F-MA11	54
AHMC type F-MA12	50
Sodium polyhydroxy aluminum monocarbonate hexitol complex	275
Aluminum dihydroxy aminoacetate	103
Aluminum dihydroxy sodium carbonate	70
Aluminum hydroxide dried gel U. S. P.-magnesium carbonate U. S. P. dry blend (4:1)	1,570

**Procedure of Holbert, Noble, and Grote Modified.**—In 1948 Holbert, Noble, and Grote (28) published an *in vitro* method which was adapted from the carefully worked-out procedure of Johnson and Duncan (21). This made use of the concept of representing the secretion of fresh gastric juice and also continuous loss of gastric juice-antacid mixture through the pylorus. Holbert, *et al.*, added a 2-Gm. dose of antacid to 150 ml. of artificial gastric juice at 37.5° with constant agitation, and after ten minutes withdrew 20 ml. of mixture and replaced it with 20 ml. of fresh artificial gastric juice. This withdrawal and replacement was carried out periodically every ten minutes until the pH fell below 3.5. Murphey (27) also used this method with an artificial gastric juice consisting of 2.0 Gm. pepsin N. F. per liter of pH 1.5 hydrochloric acid.

We have adapted this method with the following changes which we feel impart a higher degree of precision and insures a greater reproducibility of results.

A dose of antacid is added to the equivalent of 150 ml. artificial gastric juice at 37.5  $\pm$  1° contained in a

jacketed, baffled, glass vessel provided with a glass and reference electrode, thermoelectrode, agitator, and overflow device. The artificial gastric juice is pH 1.5 hydrochloric acid (0.0316 *N*) containing 2.0 Gm. pepsin N. F. per liter. After ten minutes, additional gastric juice at  $37.5 \pm 1^\circ$  is added continuously at the equivalent of 120 ml. per hour by means of a Milton Roy controlled volume "mini pump." A jacketed, 3-gallon glass reservoir of artificial gastric juice is maintained at  $37.5^\circ$  by means of an Amineco constant temperature water circulating bath. Excess gastric juice-antacid mixture is allowed to flow continuously from the glass reactor at the equivalent of 120 ml. per hour.

The apparatus designed for this purpose is shown in Figs 1 and 2. The pH is measured by means of a Beckman model W pH meter which is connected to a Weston strip chart recorder operating with a chart speed of six inches per hour. The pH meter indicates and records values between 2.0 and 12.0. In practice, the actual amounts and sizes are four times those stated above to avoid crowding into a small container. Thus, 600 ml. of artificial gastric juice are added to a jacketed, 1-liter, glass vessel provided with an overflow tube at the 650 ml. level. The agitator is a two-inch, three-bladed, marine type propeller which operates at exactly 400 r. p. m. Glass baffles prevent swirling in the reaction vessel. The pumping rate for artificial gastric juice is 480 ml.

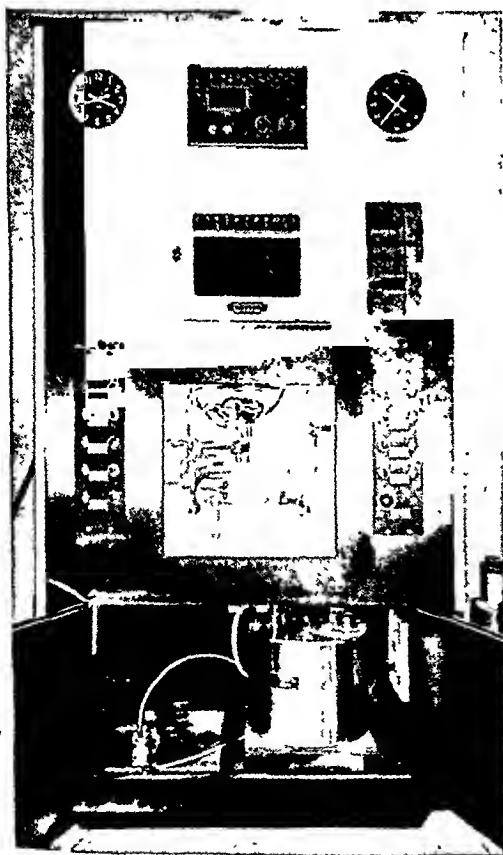


Fig. 1.—Automated apparatus for *in vitro* evaluation of antacid activity.



Fig. 2.—Close-up of cubicle showing details of glass reaction cell.

$\pm 24$  ml per hour. The overflow is also at 480 ml. per hour. The sample size is four times the dose size. After addition of the sample to the glass reaction cell, the interval timer is started and pH values manually recorded every minute up to ten minutes, after which the constant addition of gastric juice is started. With the automation provided, the apparatus needs no further attention until the run is completed, which is usually when the pH has gone below 3.0. The average run is one hundred and twenty minutes. The reproducibility of results is very high.

When evaluating dry powders, 2, 4, or 8 Gm (corresponding to doses of 0.5, 1, and 2 Gm, respectively) are dispersed in 35 ml. of water and poured in at time zero. Sucking tablets are ground to 100% through 100 mesh before dispersing in 35 ml of water. Disintegrating types of tablets are dropped into 35 ml. of water and allowed to hydrate and disintegrate before adding. Liquid antacids are added directly to the glass reaction cell.

More than 800 two-hour evaluations have been made since late 1955 including countless samples which have been submitted by various pharmaceutical manufacturers. Many samples were blind samples to insure complete objectivity. Results have been treated on a highly confidential basis. Some results obtained on various antacid chemicals are shown in Table V and plotted in Figs 3-15. Results obtained on the most widely used liquid commercial antacids are shown in Table VI, and on tablet preparations in Table VII.

**Effect of Pepsin.**—To demonstrate the effect of pepsin in artificial gastric juice on the antacid activity of dried aluminum hydroxide gel U. S. P. as measured by the modified procedure of Holbert, Noble, and Grote, runs were made on 1.0- and 2.0-Gm samples with and without pepsin. The data are shown in Table VIII and Fig. 16.

Liquid aluminum hydroxide gel is not inhibited in this manner by pepsin. The full significance of this fact is not yet understood. AHMC dried gels do not show such inhibition by gastric pepsin.

**Effect of Age.**—The antacid activity of four-year old samples of AHMC dried gel, aluminum dihydroxy aminoacetate, and aluminum dihydroxy sodium carbonate was determined by the Holbert, Noble, and Grote method on the basis of 1-Gm. doses.



TABLE V—*In Vitro* EVALUATION OF ANTACID ACTIVITY OF VARIOUS ANTACID CHEMICALS<sup>a b</sup>

Time (min)	1				2		3			4			5		
	5 ml	10 ml	12.5 ml	15 ml	1 Gm	2 Gm	0.5 Gm	1.0 Gm	2.0 Gm	0.5 Gm	1.0 Gm	2.0 Gm	0.5 Gm	1.0 Gm	2.0 Gm
0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
1	2.6	3.8	3.9	3.9	2.0	2.2	2.7	4.0	4.1	2.4	3.5	4.4	2.1	3.0	3.8
2	3.8	3.9	3.9	4.0	2.0	2.3	3.6	4.1	4.3	2.8	3.9	4.5	2.7	3.7	4.0
3	3.9	3.9	4.0	4.0	2.1	2.4	3.8	4.2	4.3	3.2	4.0	4.7	3.2	3.9	4.1
4	3.9	3.9	4.0	4.1	2.1	2.4	3.9	4.2	4.4	3.7	4.1	4.8	3.4	3.9	4.1
5	3.9	3.9	4.0	4.1	2.1	2.5	4.0	4.2	4.4	4.0	4.1	4.9	3.6	4.0	4.1
10	3.9	3.9	4.0	4.2	2.3	3.3	4.1	4.3	4.5	4.2	4.2	5.3	4.0	4.2	4.2
20	3.9	3.9	4.0	4.2	2.9	4.0	4.1	4.3	4.4	4.2	4.2	5.1	4.0	4.2	4.2
30	3.9	3.9	4.0	4.2	3.9	4.0	4.1	4.3	4.4	4.2	4.2	4.8	4.0	4.1	4.2
40	3.9	3.9	4.0	4.2	3.9	4.0	4.1	4.2	4.3	4.2	4.2	4.7	3.8	4.1	4.2
50	3.9	3.9	4.0	4.2	3.8	4.0	4.0	4.2	4.3	4.2	4.1	4.5	3.6	4.0	4.2
60	3.8	3.9	4.0	4.1	3.8	4.0	4.0	4.2	4.3	4.1	4.1	4.5	3.3	4.0	4.2
70	3.5	3.9	4.0	4.1	3.7	4.0	3.9	4.2	4.3	3.9	4.1	4.4	3.0	3.9	4.2
80	3.0	3.9	4.0	4.1	3.5	3.9	3.7	4.2	4.3	3.6	4.0	4.3	2.8	3.8	4.2
90	2.6	3.8	3.9	4.1	3.2	3.9	3.5	4.1	4.2	3.2	4.0	4.3	2.7	3.6	4.1
100	2.4	3.8	3.9	4.1	3.0	3.9	3.1	4.1	4.2	2.9	4.0	4.3	2.5	3.4	4.1
110	2.2	3.7	3.9	4.1	2.8	3.8	2.8	4.1	4.2	2.7	3.9	4.2	2.4	3.2	4.1
120	2.1	3.4	3.8	4.1	2.6	3.7	2.6	4.0	4.2	2.5	3.9	4.2	2.3	3.0	4.0

Time min	6			7			8		9			10		
	0.5 Gm	1.0 Gm	2.0 Gm	0.5 Gm	1.0 Gm	2.0 Gm	0.5 Gm	1.1 Gm	0.5 Gm	1.0 Gm	2.0 Gm	0.5 Gm	1.0 Gm	2.0 Gm
0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
1	3.1	3.8	4.2	2.0	2.0	2.3	2.3	3.6	2.0	2.0	2.0	6.6	7.2	7.6
2	3.5	4.0	4.3	2.0	2.0	2.5	2.9	3.8	2.0	2.2	2.3	6.9	7.6	7.8
3	3.6	4.1	4.4	2.0	2.0	2.7	3.5	3.9	2.0	2.6	2.9	7.1	7.8	7.9
4	3.7	4.1	4.5	2.0	2.1	2.9	3.7	4.0	2.0	4.1	4.2	7.2	7.9	8.0
5	3.8	4.2	4.5	2.1	2.2	3.0	3.7	4.0	2.6	5.3	5.7	7.8	8.0	8.0
10	3.9	4.3	4.9	2.4	2.5	3.2	3.9	4.0	2.2	6.8	7.2	7.2	7.7	7.8
20	3.6	4.2	4.6	2.5	2.8	3.2	3.8	4.0	2.4	6.7	7.2	7.6	7.6	7.8
30	3.2	4.1	4.3	2.3	2.8	3.1	3.7	4.0	2.4	6.6	7.1	7.0	7.6	7.8
40	3.1	4.0	4.3	2.3	2.7	3.0	3.7	4.0	2.3	6.3	7.1	6.8	7.6	7.7
50	3.0	3.9	4.2	2.3	2.7	3.0	3.6	3.9	2.2	5.8	7.0	6.5	7.5	7.7
60	2.9	3.9	4.2	2.2	2.6	2.9	3.5	3.9	2.1	4.8	7.0	6.2	7.4	7.7
70	2.8	3.8	4.2	2.1	2.6	2.9	3.2	3.9	2.1	3.9	6.9	5.3	7.3	7.7
80	2.6	3.8	4.1	2.1	2.5	2.8	2.8	3.8	2.1	3.2	6.7	3.0	7.1	7.6
90	2.4	3.7	4.1	2.1	2.4	2.7	2.5	3.8	2.0	2.7	6.5	2.6	6.9	7.5
100	2.3	3.6	4.0	2.0	2.3	2.7	2.3	3.7	2.0	2.4	6.3	2.4	6.7	7.5
110	2.2	3.6	4.0	2.0	2.3	2.6	2.2	3.5	2.0	2.3	5.6	2.3	6.4	7.4
120	2.1	3.5	4.0	2.0	2.2	2.5	2.1	3.4	2.0	2.2	5.6	2.2	5.8	7.3

Time min	11			12			13		
	0.5 Gm	1.0 Gm	2.0 Gm	0.5 Gm	1.0 Gm	2.0 Gm	0.5 Gm	1.0 Gm	2.0 Gm
0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
1	6.2	8.9	9.1	5.7	5.8	5.9	5.4	6.4	6.9
2	7.2	9.0	9.1	5.8	5.8	6.0	5.5	6.4	7.0
3	8.6	9.0	9.2	5.8	5.8	6.0	5.5	6.5	7.0
4	8.7	9.1	9.2	5.9	5.9	6.0	5.5	6.5	7.0
5	8.8	9.1	9.2	5.9	5.9	6.0	5.5	6.5	7.1
10	8.9	9.1	9.2	6.0	6.0	6.1	5.6	6.7	7.2
20	8.9	8.9	9.1	5.9	6.0	6.1	4.5	6.7	7.3
30	7.8	8.8	9.1	5.9	6.0	6.1	2.8	6.7	7.2
40	7.6	8.8	9.1	5.9	6.0	6.1	2.5	6.6	7.2
50	7.3	8.7	9.1	5.8	6.0	6.1	2.3	6.5	7.2
60	6.9	8.6	9.0	5.7	6.0	6.1	2.2	6.2	7.2
70	6.4	8.5	9.0	4.7	6.0	6.1	2.1	5.3	7.2
80	5.6	8.3	8.9	3.2	5.9	6.1	2.1	2.9	7.1
90	4.9	8.2	8.9	2.8	5.9	6.1	2.0	2.6	7.0
100	4.4	8.0	8.9	2.5	5.8	6.1	2.0	2.6	6.8
110	3.9	7.7	8.8	2.3	5.8	6.1	2.0	2.2	6.5
120	3.2	7.4	8.7	2.2	5.7	6.0	2.0	2.2	6.3

<sup>a</sup> Time versus pH at various dose levels procedure of Holbert, Noble, and Grote modified<sup>b</sup> Identification of samples

- 1 Aluminum hydroxide gel U S P
- 2 Aluminum hydroxide dried gel U S P
- 3 AHMC type F MA11
- 4 AHMC type F MA12
- 5 Aluminum dihydroxy aminoacetate
- 6 Aluminum dihydroxy sodium carbonate
- 7 Aluminum proteinate
- 8 Sodium polyhydroxy aluminum monocarbonate bexitol complex
- 9 Magnesium trisilicate U S P
- 10 Magnesium carbonate U S P
- 11 Magnesium hydroxide N F
- 12 Calcium carbonate U S P
- 13 Sodium bicarbonate U S P

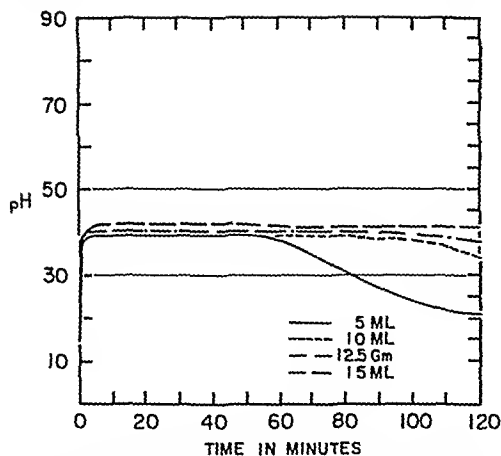


Fig 3—Aluminum hydroxide gel U. S. P. Procedure of Holbert, Noble, and Grote modified.

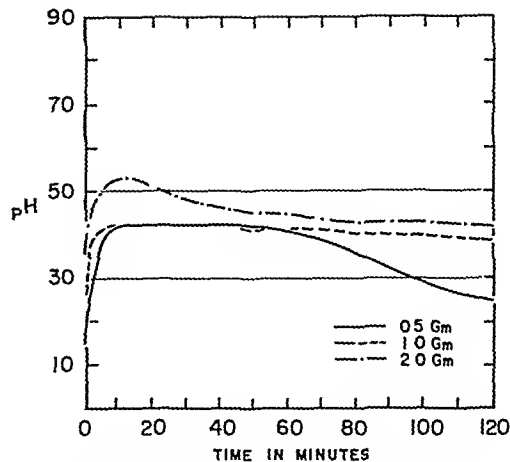


Fig 6.—AHMC type F-MA12, procedure of Holbert, Noble, and Grote modified.

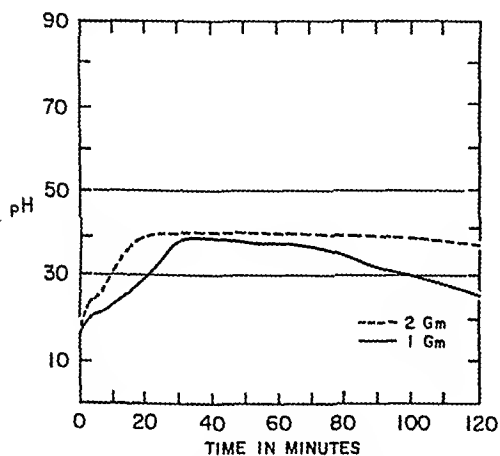


Fig 4—Aluminum hydroxide dried gel U. S. P., procedure of Holbert, Noble, and Grote modified

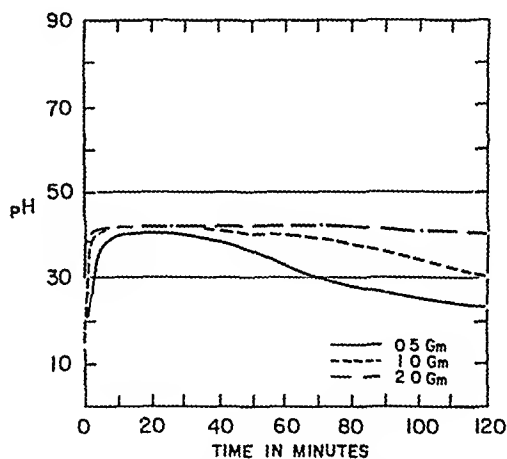


Fig 7—Aluminum dihydroxy aminoacetate, procedure of Holbert, Noble, and Grote modified.

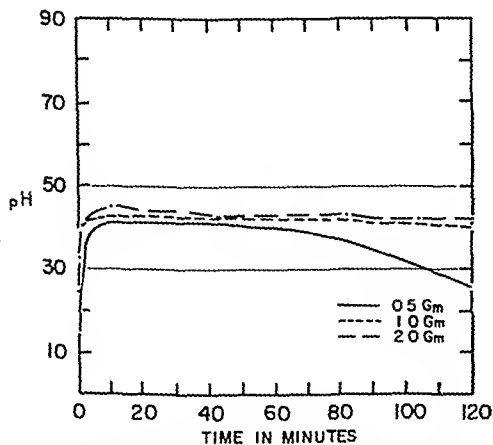


Fig. 5.—AHMC type F-MA11, procedure of Holbert, Noble, and Grote modified.

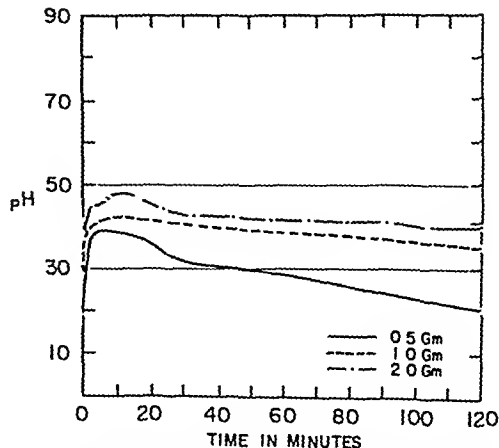


Fig. 8.—Aluminum dihydroxy sodium carbonate, procedure of Holbert, Noble, and Grote modified.

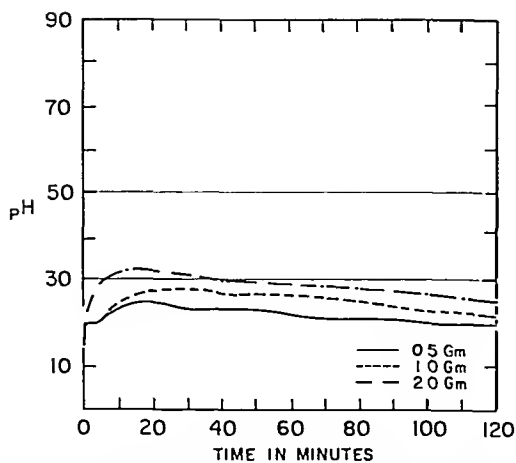


Fig. 9.—Aluminum proteinate, procedure of Holbert, Noble, and Grote modified.

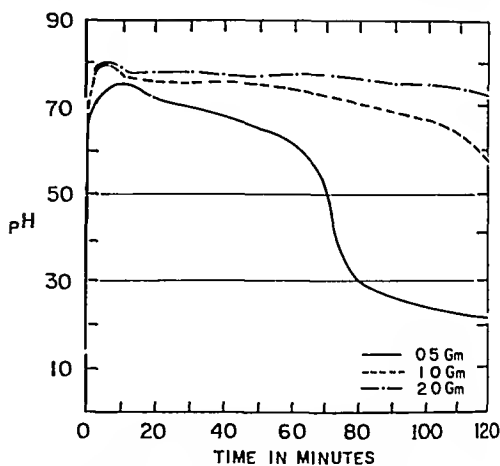


Fig. 12.—Magnesium carbonate U. S. P., procedure of Holbert, Noble, and Grote modified.

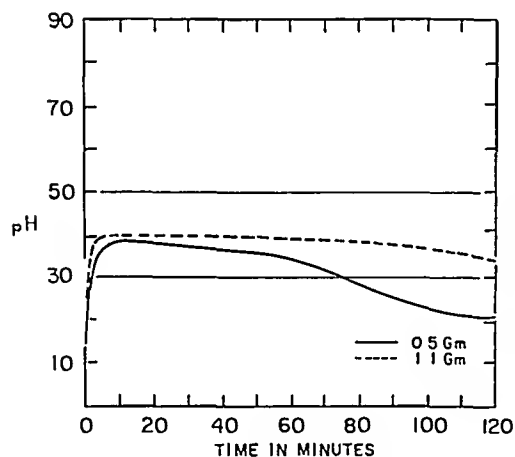


Fig. 10.—Sodium polyhydroxy aluminum mono-carbonate Ilexitol complex, procedure of Holbert, Noble, and Grote modified

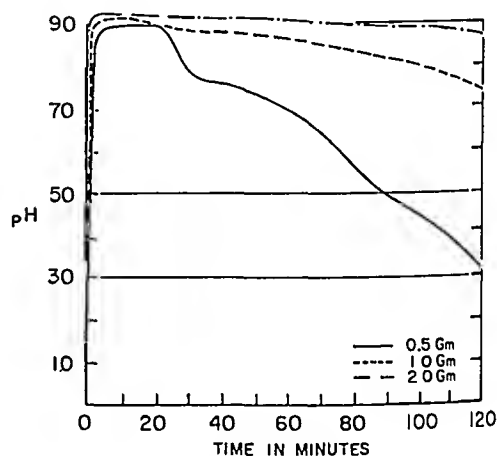


Fig. 13.—Magnesium hydroxide N. F., procedure of Holbert, Noble, and Grote modified.

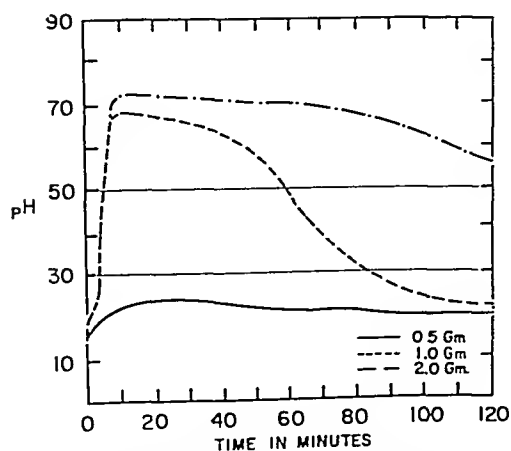


Fig. 11.—Magnesium trisilicate U. S. P., procedure of Holbert, Noble, and Grote modified.

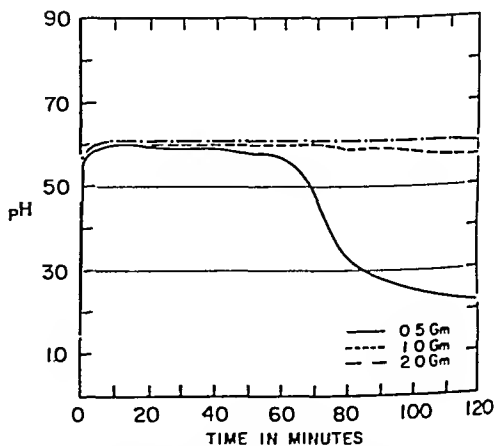


Fig. 14.—Calcium carbonate, procedure of Holbert, Noble, and Grote modified.

TABLE VI—*In Vitro* EVALUATION OF VARIOUS COMMERCIAL LIQUID ANTACIDS<sup>a b</sup>

Time, min	14 15 ml	15 15 ml	16 15 ml	4 ml	17— 8 ml	15 ml	18 15 ml	19 15 ml	20 15 ml	21— 5 ml	10 ml	22 4 ml	23 25 ml
	15	15	15	4	8	15	15	15	15	5	10	4	25
0	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5
1	2 6	4 0	3 9	2 1	3 0	3 8	5 6	4 2	3 9	2 7	3 7	2 0	3 0
2	3 1	4 1	4 0	2 5	3 3	4 0	5 7	4 2	4 0	3 3	3 9	2 0	3 0
3	3 5	4 1	4 1	2 7	3 4	4 1	5 7	4 3	4 0	3 8	4 0	2 0	3 1
4	3 7	4 1	4 1	2 9	3 5	4 2	5 8	4 3	4 0	3 9	4 1	2 0	3 1
5	3 8	4 1	4 1	3 0	3 6	4 3	5 8	4 3	4 1	4 0	4 1	2 0	3 1
10	4 1	4 1	4 2	3 2	3 8	4 6	5 9	4 4	4 1	4 1	4 2	2 0	3 2
20	4 1	4 1	4 2	3 0	3 6	4 3	5 8	4 2	4 1	4 2	4 2	2 0	3 0
30	4 2	4 0	4 2	2 8	3 5	4 1	5 7	4 2	4 0	4 3	4 2	2 0	2 9
40	4 2	4 0	4 3	2 7	3 4	3 9	5 5	4 1	4 0	4 3	4 3	2 0	2 9
50	4 2	4 0	4 3	2 5	3 2	3 7	5 4	4 1	3 9	4 3	4 3	2 0	2 8
60	4 1	4 0	4 3	2 3	3 1	3 6	5 2	4 1	3 9	4 2	4 3	2 0	2 8
70	4 1	3 9	4 3	2 2	2 9	3 5	5 0	4 0	3 8	4 1	4 3	2 0	2 8
80	4 0	3 9	4 3	2 1	2 7	3 4	4 7	4 0	3 6	4 0	4 2	2 0	2 7
90	3 9	3 9	4 2	2 1	2 5	3 4	4 5	4 0	3 5	4 0	4 2	2 0	2 7
100	3 7	3 8	4 0	2 0	2 4	3 3	4 4	3 9	3 5	3 8	4 1	2 0	2 7
110	3 5	3 8	3 9	2 0	2 3	3 3	4 3	3 8	3 3	3 6	4 0	2 0	2 6
120	3 0	3 7	3 8	2 0	2 2	3 2	4 2	3 7		3 3	3 9	2 0	2 6

<sup>a</sup> Time versus pH at various dose levels procedure of Holbert Noble, and Grote modified<sup>b</sup> Identification of samples

- 14 Aluminum hydroxide gel U S P, brand A  
 15 Aluminum hydroxide gel U S P, brand B  
 16 Aluminum-magnesium-magnesium hydroxide  
 17 Aluminum trisilicate  
 18 Aluminum  
 19 Magnesium aluminum hydroxide (322 mg) per 5 ml  
 20 Aluminum hydroxide gel with magnesium hydroxide  
 21 Aluminum hydroxide gel-magnesium hydroxide-sorbitol  
 22 Bismuth aluminate cream  
 23 Aluminum phosphate gel U S P

TABLE VII—*In Vitro* EVALUATION OF VARIOUS COMMERCIAL ANTACID TABLETS<sup>a b</sup>

Time, min	24		25		26		27		28		29	
	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab
0	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5
1	2 0	2 0	2 0	2 0	2 2	3 8	2 8	3 5	3 5	2 0	2 7	4 1
2	2 0	2 1	2 0	2 2	3 3	4 0	3 4	3 9	3 8	2 0	3 3	5 0
3	2 0	2 1	2 0	2 4	3 7	4 1	3 6	4 1	3 9	2 4	3 7	5 5
4	2 0	2 2	2 1	2 6	3 8	4 1	3 9	4 1	3 9	2 6	3 8	5 7
5	2 0	2 3	2 1	2 8	3 8	4 1	3 9	4 2	4 0	2 7	4 0	5 9
10	2 4	3 0	2 3	3 4	3 9	4 2	4 0	4 2	4 1	2 9	5 2	6 1
20	3 5	3 7	2 3	3 3	4 0	4 2	3 9	4 2	4 2	2 8	4 6	5 9
30	3 5	3 7	2 3	3 1	4 0	4 2	3 8	4 2	4 2	2 6	4 2	5 7
40	3 5	3 7	2 2	2 9	4 0	4 2	3 6	4 2	4 2	2 4	3 8	5 6
50	3 5	3 7	2 2	2 7	4 0	4 1	3 4	4 2	4 1	2 3	3 4	5 4
60	3 4	3 7	2 1	2 6	4 0	4 1	3 0	4 1	4 1	2 2	3 3	5 2
70	3 4	3 6	2 1	2 4	3 9	4 1	2 8	4 1	4 1	2 2	3 1	4 9
80	3 3	3 5	2 0	2 3	3 7	4 1	2 6	3 9	4 0	2 1	3 0	4 6
90	3 2	3 5	2 0	2 2	3 4	4 1	2 5	3 7	3 9	2 0	2 9	4 1
100	3 1	3 3	2 0	2 1	2 9	4 0	2 3	3 5	3 6	2 0	2 7	3 7
110	3 0	3 1	2 0	2 1	2 6	4 0	2 2	3 2	3 3	2 0	2 6	3 3
120	2 9	3 0	2 0	2 0	2 4	4 0	2 1	2 9	3 1	2 0	2 5	3 0

Time min	30		31		32		33		34		35		36	
	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab	1 Tab	2 Tab
0	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5
1	2 0	3 0	3 6	2 0	2 8	2 0	2 8	3 5	2 4	3 6	5 3	3 5	4 0	5 2
2	2 3	3 8	3 8	2 1	3 4	2 0	3 4	4 0	2 9	3 9	6 2	3 6	4 1	5 3
3	2 6	3 9	3 9	2 2	3 7	2 1	3 7	4 2	3 2	4 0	6 6	3 7	4 1	5 4
4	2 7	4 0	4 0	2 3	3 8	2 1	3 9	4 3	3 4	4 0	6 9	3 8	4 2	5 4
5	3 2	4 0	4 0	2 5	3 9	2 1	4 0	4 5	3 5	4 1	7 1	4 0	4 2	5 5
10	3 8	4 0	4 0	3 2	4 0	2 5	4 2	6 7	3 8	4 3	7 6	4 1	4 2	5 6
20	3 6	4 0	4 0	3 4	4 0	2 8	4 3	7 1	3 8	4 4	7 8	3 9	4 1	5 2
30	3 4	4 0	4 0	3 5	4 0	2 8	4 3	6 8	3 7	4 3	7 7	3 6	4 0	5 2
40	3 0	4 0	4 0	3 3	4 0	2 8	4 2	6 4	3 6	4 2	7 5	3 3	4 0	5 0
50	2 6	4 0	3 9	3 1	3 9	2 8	4 1	5 7	3 5	4 0	7 1	3 1	3 9	4 7
60	2 4	3 9	3 9	2 9	3 9	2 8	4 0	4 9	3 4	3 9	6 5	2 9	3 8	4 2
70	2 2	3 9	3 9	2 7	3 8	2 7	3 9	4 4	3 3	3 8	5 9	2 7	3 6	3 1
80	2 1	3 8	3 8	2 6	3 7	2 5	3 8	4 2	3 2	3 8	5 2	2 6	3 5	2 6
90	2 0	3 6	3 7	2 5	3 6	2 4	3 8	4 0	3 0	3 7	4 6	2 4	3 3	2 4
100	2 0	3 3	3 7	2 4	3 5	2 4	3 7	3 8	2 9	3 7	4 3	2 3	3 2	2 2
110	2 0	2 9	3 5	2 3	3 3	2 3	3 6	3 7	2 7	3 6	4 0	2 2	3 0	2 1
120	2 0	2 7	3 4	2 2	3 1	2 3	3 5	3 6	2 6	3 5	3 8	2 1	2 9	2 1

TABLE VII (continued)

Time, min	37	38			39		40		41	42	43		
	Tab	1 Tab	2 Tab	4 Tab	1 Tab	2 Tab	1 Tab	2 Tab	2 Tab	2 Tab	1 Tab	2 Tab	4 Tab
0	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1 5	1.5	1 5	1 5	1 5
1	6 0	4 5	5 7	6 2	5 5	6 0	5 4	6 0	2 2	3 5	3 9	4 1	4 3
2	6 2	5 1	6 2	7 1	5 7	6 3	5 7	6 2	2 8	3 8	4 0	4 3	4 8
3	6 3	5 4	6 6	7 9	5 8	6 5	5 9	6 4	3 3	3 9	4 1	4 4	5 2
4	6 3	5 6	7 0	8 2	5 9	6 6	6 0	6 4	3 9	3 9	4 1	4 6	5 4
5	6 4	5 7	7 5	8 3	6 0	6 7	6 1	6 5	4 4	3 9	4 1	4 7	5 6
10	6 5	6 1	8 2	8 6	6 2	7 1	6 3	6 7	5 5	4 0	4 1	5 1	5 8
20	6 3	5 7	7 9	8 4	6 9	6 1	6 3	6 5	5 2	4 0	4 0	4 5	4 9
30	6 2	5 0	7 6	8 3	5 9	6 7	6 2	6 5	4 5	4 0	3 9	4 3	4 8
40	6 2	4 3	7 1	8 1	5 8	6 4	6 1	6 5	3 9	4 0	3 9	4 2	4 7
50	6 2	3 6	6 7	7 9	5 7	6 3	6 0	6 5	3 5	3 9	3 8	4 1	4 5
60	6 2	3 0	6 3	7 7	5 4	6 1	5 7	6 4	3 3	3 9	3 6	4 1	4 4
70	6 1	2 6	5 9	7 5	4 9	6 0	4 9	6 4	2 9	3 9	3 2	4 0	4 3
80	6 0	2 5	5 4	7 2	4 6	6 0	4 0	6 3	2 7	3 8	2 8	4 0	4 2
90	5 9	2 3	4 7	7 0	4 1	5 8	3 7	6 2	2 5	3 7	2 5	3 9	4 2
100	5 7	2 2	4 0	6 7	3 6	5 7	3 5	6 1	2 4	3 5	2 3	3 8	4 1
110	5 3	2 2	3 3	6 5	3 1	5 5	3 3	5 8	2 3	3 3	2 2	3 7	4 0
120	4 2	2 1	2 9	6 2	2 8	5 2	3 1	5 1	2 2	3 0	2 1	3 5	4 0

a Time versus pH at various dose levels, procedure of Holbert, Noble and Grote modified

b Identification of samples

- 24 Aluminum hydroxide dried gel (660 mg )  
 25 Aluminum hydroxide dried gel (660 mg )  
 26 AHMC type F MA11 (660 mg )  
 27 Aluminum dibydroxy aminoacetate (500 mg )  
 28 Magnesium trisilicate (500 mg ) and aluminum hydroxide (250 mg )  
 29 Aluminum-magnesium hydroxide (400 mg )  
 30 Sodium polyhydroxy aluminum monocarbonate hexitol complex (320 mg ) calculated as aluminum hydroxide dried gel  
 U S P  
 31 Aluminum hydroxide (648 mg ) and magnesium trisilicate (324 mg )  
 32 Aluminum hydroxide and magnesium trisilicate  
 33 Aluminum hydroxide-glycine (450 mg ), magnesium oxide (60 mg ), and bellafoline (0.5 mg )  
 34 Aluminum hydroxide-glycine (450 mg ) and magnesium oxide (60 mg )  
 35 Aluminum dihydroxy sodium carbonate (330 mg )  
 36 Calcium carbonate (510 mg ), magnesium carbonate (30 mg ), and magnesium trisilicate (40 mg )  
 37 Calcium carbonate, magnesium carbonate, and magnesium trisilicate  
 38 Calcium carbonate, magnesium trisilicate, and magnesium hydroxide  
 39 Calcium carbonate, magnesium carbonate, and magnesium trisilicate  
 40 Heavy magnesium carbonate (182 mg ) light magnesium carbonate (24 mg ), calcium carbonate (441 mg ), and aluminum hydroxide (223 mg )  
 41 Regenal (100 mg ) magnesium trisilicate (100 mg ), aluminum hydroxide dried gel (90 mg ), calcium carbonate (100 mg ), magnesium carbonate (100 mg ), and egraine (45 mg )  
 42 AHMC type F MA11 (330 mg ) and activated attapulgit (130 mg )  
 43 Sodium polyhydroxy aluminum monocarbonate bexitol complex (320 mg ), calculated as aluminum hydroxide dried gel  
 U S P, and magnesium hydroxide (75 mg )

TABLE VIII—EFFECT OF PEPSIN ON ANTACID ACTIVITY OF DRIED ALUMINUM HYDROXIDE GEL U S P, TIME versus pH

Time, min	Concentration of Pepsin—			
	2.0 Gm/L	2 Gm	0.0 Gm/L	2 Gm
0	1 5	1 5	1 5	1 5
1	2 0 <sup>a</sup>	2 1	2 0 <sup>a</sup>	2 2
3	2 0 <sup>a</sup>	2 2	2 0 <sup>a</sup>	2 4
5	2 0 <sup>a</sup>	2 3	2 0	2 5
10	2 0	2 6	3 9	4 2
20	2 2	3 3	4 1	4 2
30	2 4	4 0	4 2	4 2
40	2 6	4 0	4 2	4 2
50	2 7	4 0	4 2	4 2
60	2 8	4 0	4 2	4 2
70	2 8	4 0	4 2	4 2
80	2 7	3 9	4 2	4 2
90	2 6	3 9	4 2	4 2
100	2 6	3 8	4 2	4 2
110	2 5	3 7	4 2	4 1
120	2 4	3 5	4 1	4 1

c Indicates less than

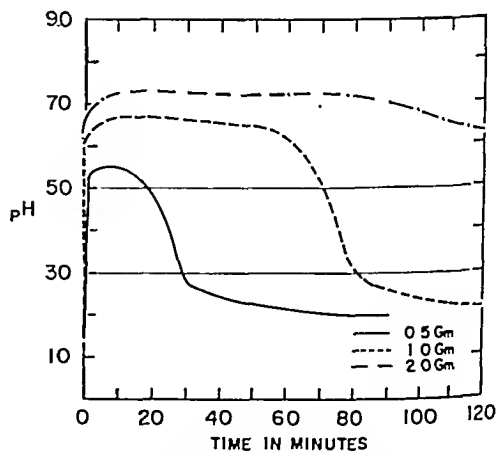


Fig. 15—Sodium bicarbonate U. S. P., procedure of Holbert, Noble, and Grote modified.

The results, which are plotted in Fig. 17, show that AHMC is somewhat more reactive after four years storage at ambient temperatures than the other two samples.

**Comparison with Dry Blend.**—To demonstrate the difference in antacid properties between the

new AHMC dried gels and a simple dry blend of aluminum hydroxide dried gel U. S. P. and magnesium carbonate U. S. P. having an  $\text{Al}_2\text{O}_3:\text{MgO}$  molar ratio of 2:1, the following experiment was performed. Four parts of dried aluminum hydroxide was thoroughly blended with one part of magnesium

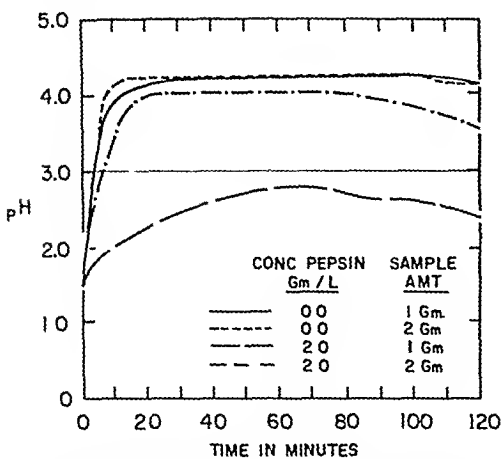


Fig. 16.—Effect of pepsin on antacid activity of dried aluminum hydroxide gel U. S. P., procedure of Holbert, Noble, and Grote modified.

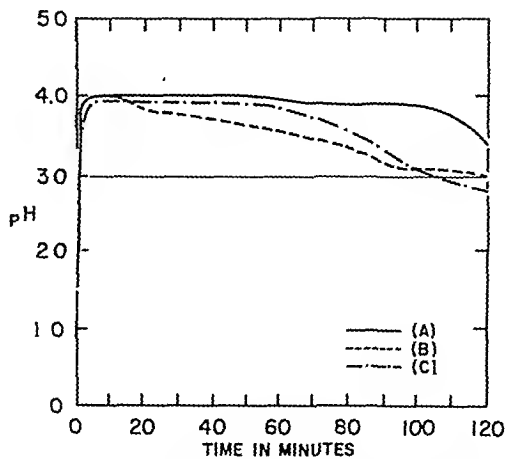


Fig. 17.—Antacid activity of four-year old samples of three aluminum antacids: (A) AHMC; (B) aluminum dihydroxy sodium carbonate; (C) aluminum dihydroxy aminoacetate; procedure of Holbert, Noble, and Grote modified.

carbonate, and the antacid activity of the blend was determined on the basis of 1 Gm. and 2 Gm. The data are shown in Table IX and plotted in Fig. 18.

TABLE IX.—ANTACID ACTIVITY COMPARISON OF AHMC WITH ALUMINUM HYDROXIDE-MAGNESIUM CARBONATE DRY BLEND (4:1)<sup>a</sup>

Time, min	AHMC		Aluminum Hydroxide-Magnesium Carbonate Dry Blend (Mole ratio—Al <sub>2</sub> O <sub>3</sub> : MgO—2:1)	
	1 Gm	2 Gm	1 Gm	2 Gm
0	1.5	1.5	1.5	1.5
1	4.0	4.1	4.5	5.8
2	4.1	4.3	4.5	6.4
3	4.2	4.3	4.6	6.6
4	4.2	4.4	4.7	6.8
5	4.2	4.4	4.7	6.9
10	4.3	4.5	4.8	7.2
20	4.3	4.4	3.8	6.9
30	4.3	4.4	3.1	6.6
40	4.2	4.3	2.8	6.2
50	4.2	4.3	2.7	5.7
60	4.2	4.3	2.6	4.6
70	4.2	4.3	2.5	3.7
80	4.2	4.3	2.4	3.1
90	4.1	4.2	2.4	2.7
100	4.1	4.2	2.3	2.5
110	4.1	4.2	2.3	2.4
120	4.0	4.2	2.3	2.3

<sup>a</sup> Time versus pH, procedure of Holbert, Noble, and Grote

### RESULTS

The data in Tables III and IV show that by both reaction velocity tests AHMC dried gels are rapidly reactive with 0.1 N HCl at 37.5° and compare favorably with a highly reactive aluminum hydroxide gel U. S. P. (prepared from Reheis F-500 aluminum hydroxide compressed gel). Aluminum dihydroxy sodium carbonate, which is also shown to be rapidly reactive by the Reheis reaction velocity test, contains 306 mg. of sodium oxide for each 500 mg. of aluminum oxide. Aluminum dihydroxy aminoacetate and sodium polyhydroxy aluminum monocarbonate hexitol complex are only slightly less reactive than AHMC.

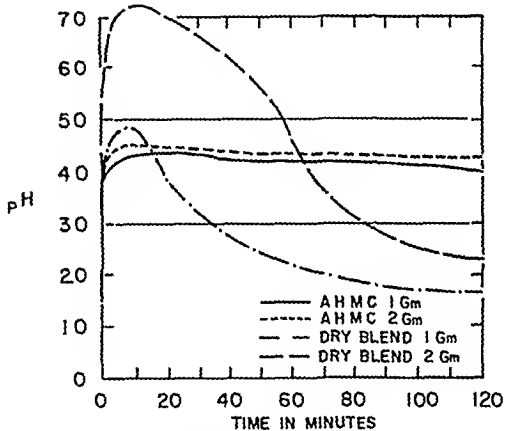


Fig. 18.—Comparison of antacid activity of AHMC with aluminum hydroxide-magnesium carbonate dry blend (4:1), procedure of Holbert, Noble, and Grote modified.

The data in Table V, which are plotted in Figs. 3-15, show that both types of AHMC dried gel are very rapid and prolonged in their antacid activity in the pH range of 3 to 5 with artificial gastric juice. In this respect, they compare favorably with the ideal, a reactive liquid aluminum hydroxide gel.

The data plotted in Fig. 17 shows that AHMC retains this high degree of reactivity over a long period of time. Not a single substance tested shows better results than the AHMC dried gels. Table VII shows that the superior antacid characteristics of AHMC is retained in tablets as well. Hinkel, Fisher, and Tainter (25, 26) described a new highly reactive aluminum hydroxide (sodium polyhydroxy aluminum monocarbonate hexitol complex) which, when blended with magnesium hydroxide, forms the basis of an improved antacid tablet. The data for sample 43, Table VII indicates that the antacid characteristics of this new improved tablet are excellent and compare favorably with AHMC powder and tablets.

## DISCUSSION

A new combination of two old antacids, aluminum hydroxide and magnesium carbonate, is shown to possess outstanding antacid properties. The principal method employed in this *in vitro* study is a very stringent test. It was Johnson and Duncan who first introduced the periodic replacement of antacid-acid mixture with the fresh gastric acid. They used an equivalent dose, which varied for each product tested, depending upon the acid neutralizing power of the substance. Holbert, Noble, and Grote (28) used a 2-Gm. sample and employed an artificial gastric juice instead of 0.1 N HCl. Our modification consisted of (a) establishing an exact normality for the artificial gastric juice and (b) replacing it at a constant rate. The rate of change of pH with time during the first ten minutes is a function of its reactivity and, hence, the test is unexcelled for measuring promptness of buffering. Since, at the ten-minute interval an excess of antacid is usually present, the pH at ten minutes is a measure of the maximum pH that might be expected. The concentration of acid (pH 1.5–0.0316 N) in the artificial gastric juice also makes the test a very stringent one. A substance which reacts well with acid of this strength will react even more rapidly with a stronger acid. The constant replacement of reaction mixture with fresh gastric juice provides a system where the amount of antacid is gradually diminishing while the supply of fresh acid is constant. A more stringent test for an antacid substance cannot be devised. One, of course, may vary the concentration and composition of the gastric juice and obtain a whole family of curves and data which is constant for the new gastric juice. We have used simulated gastric fluid U S P, which became official after our investigation was started. This is approximately 0.08 N but may vary depending on the concentration of hydrochloric acid used. Our results with this more concentrated gastric juice show that the total time in the pH range of 3 to 5 is less, but that the promptness of reaction and duration of action is similar for AHMC dried gel and liquid aluminum hydroxide gel.

The *in vitro* procedure, as employed, by us is not represented as being a duplication of the behavior of an antacid in the human stomach. It is perhaps the most severe method of comparing antacids that can be devised under conditions similar to those found in the stomach. It is doubtful whether any *in vivo* test so far described is fully adequate to differentiate between a large number of antacids. The final and true test must remain in the hands of competent clinicians.

**Nature of Aluminum Hydroxide Gel.**—Reactive aluminum hydroxide gel is a very dynamic system whose properties are dependent on the presence of immobilized watersheds and the nature and amount of absorbed foreign anions and cations. The loss of protons, which are expelled from the hydration shells as a result of electrostatic attraction between an aluminum ion and an hydroxyl group in a neighboring hydration shell, brings about a polymerization. This structure can be stabilized by substances which help to retain the remaining protons in the hydration shells. Submicroscopic particles of magnesium carbonate is an excellent stabilizer of this structure.

## SUMMARY

1. The preparation and properties of two new aluminum hydroxide–magnesium carbonate dried gels have been described.

2. *In vitro* evaluation of the antacid properties of AHMC dried gels by four methods demonstrate that its buffering action is very rapid and prolonged in the optimum pH range of 3 to 5. It compares favorably in this respect with reactive liquid aluminum hydroxide gels.

3. The outstanding antacid properties of AHMC are retained over a long period of time.

4. Of all other antacid chemicals tested in powder form, only aluminum dihydroxy aminoacetate, aluminum dihydroxy sodium carbonate, and sodium polyhydroxy aluminum mono-carbonate hexitol complex exhibited prompt and prolonged activity in the pH range of 3 to 5.

5. *In vitro* antacid evaluation data on 30 of the most widely used antacids in liquid and tablet form are included for purposes of comparison.

## REFERENCES

- (1) Einsel, I. H., Adams, W. L., and Myers, V. C., *Am J Digest Diseases*, **1**, 513(1934).
- (2) Woldman, E. E., and Rowland, V. C., *Ret Gastroenterol*, **3**, 27(1936).
- (3) Emery, F. S., Jr., and Rutherford, R. B., *Am J Digest Diseases*, **5**, 186(1938).
- (4) Eads, J. T., *ibid*, **7**, 32(1910).
- (5) Jones, C. R., Jr., *Am J Digest Diseases Nutrition*, **4**, 99(1937).
- (6) Connelly, A., and Hollander, F., *Rev Gastroenterol*, **9**, 351(1912).
- (7) Kygel, E. R., Jr., Hashinger, E. H., and Wilhelm, E. W., *Am J Digest Diseases*, **6**, 363(1939).
- (8) Kunzler, M. B., *Med Record*, **149**, 268(1939).
- (9) Bennett, T. I., and Gill, A. M., *Lancet*, **1**, 500(1939).
- (10) Steigmann, F., *Illinois Med J*, **76**, 113(1939).
- (11) Woldman, E. E., and Polan, C. G., *Am J Med Sci*, **198**, 155(1939).
- (12) Fauley, G. B., Ivy, A. C., Terry, L., and Bradley, W. B., *Am J Digest Diseases*, **5**, 792(1939).
- (13) Cornell, A., Hollander, F., and Winkelstein, A., *ibid*, **9**, 332(1912).
- (14) McIntosh, J. F., and Sutherland, C. G., *Can. Med Assoc J*, **42**, 110(1910).
- (15) Hardt, L. I., and Brodt, I. P., *Arch Surg*, **55**, 581(1917).
- (16) Hardt, L. I., and Steigmann, F., *Am J Digest Diseases*, **17**, 195(1950).
- (17) Morrison, S., *Am J Gastroenterol*, **21**, 301(1951).
- (18) Collins, E. N., *J Am Med Assoc*, **127**, 899(1951).
- (19) Rossett, N. E., and Flexner, J., *Ann Internal Med*, **21**, 119(1911).
- (20) Rossett, N. E., and Flexner, J., *ibid*, **18**, 193(1911).
- (21) Johnson, E. H., and Duncan, J., *Quart J. Pharm and Pharmacol*, **18**, 251(1915).
- (22) Hammarlund, E. R., and Rising, L. W., *This Jour Nat*, **38**, 586(1919).
- (23) Dale, J. K., and Booth, R. E., *ibid*, **44**, 170(1955).
- (24) Gwalt, J. R., Livingston, J. L., and Robertson, A., *J Pharm and Pharmacol*, **10**, 770(1958).
- (25) Hunkel, E. T., Jr., Fisher, M. P., and Tainter, M. L., *This Journal*, **48**, 350(1959).
- (26) Hunkel, E. T., Jr., Fisher, M. P., and Tainter, M. L., *ibid*, **48**, 381(1959).
- (27) Murphy, R. S., *ibid*, **41**, 361(1952).
- (28) Holbert, J. M., Noble, N., and Grote, I. W., *ibid*, **37**, 292(1918).
- (29) Holbert, J. M., Noble, N., and Grote, I. W., *ibid*, **36**, 119(1917).
- (30) Schenk, J., *Schweiz med Wochschr*, **84**, 1118(1954).
- (31) Muir, N., *Quart J. Pharm. and Pharmacol*, **19**, 490(1916).

# Preparation and Properties of New Gastric Antacids II\*

## Aluminum Hydroxide-Protein Dried Gels

By STEWART M. BEEKMAN and CARL H. VOGEL

Seven new antacids are described. Each is a combination of aluminum hydroxide and protein in dry form. The *in vitro* technique (based on the procedure of Holbert, Noble, and Grote) described in the first paper of this series is used to evaluate the antacid properties of the new dried gels. They are shown to be prompt in raising the pH to about 4 and prolonged in maintaining the pH in the optimum range of 3 to 5. All are palatable and free from chalky taste. The compositions include combinations of aluminum hydroxide with nonfat milk, milk protein concentrate, whole milk, egg albumin, lactalbumin, gelatin, and a soya milk product.

IN THE FIRST paper (1) in this series of studies, two new aluminum hydroxide-magnesium carbonate dried gels (AHMC) were described and their excellent antacid characteristics were determined by a modified *in vitro* procedure of Holbert, Noble, and Grote (2). A specially designed apparatus for evaluating antacid activity was also described.

The purpose of this paper is to describe several new aluminum hydroxide-protein dried gels and to present *in vitro* antacid evaluation data using the same procedure and apparatus.

Several investigators have endeavored to prepare effective antacids by combining proteins with aluminum. Lipschitz (3) proposed a blend of 29.2 parts of aluminum hydroxide dried gel with 400 parts of calcium caseinate and 100 parts of calcium carbonate. Horlicks (4) mixed 10 parts of aluminum hydroxide dried gel with 38 parts of malted milk. Paterson (5) reacted a soluble protein with aluminum ions or gel particles under conditions to precipitate an aluminum proteinate. Our evaluation of these products shows that they do not meet the requirements for a successful, modern antacid in dry form.

### EXPERIMENTAL

The antacids which are the subject of this study were all prepared by intimately contacting an aqueous solution of soluble, edible protein with a highly reactive basic aluminum carbonate gel at a pH sufficiently high to prevent precipitation, followed by desiccation and reduction to finely divided form. The proteins used were nonfat milk, milk protein concentrates, soluble lactalbumin, whole milk, egg albumin, gelatin, and a milk substitute containing soya protein. The products were white, odorless dried gels having a pleasant, nonchalky, and bland taste. In Table I the composition of several aluminum hydroxide-protein dried gels are shown.

In Table II the analyses of the various samples as well as their acid consuming capacities can be found.

TABLE I.—COMPOSITION OF VARIOUS ALUMINUM HYDROXIDE-PROTEIN DRIED GELS

Designation	Composition
ALUMIL <sup>a</sup> F-21	Al(OH) <sub>3</sub> -Nonfat milk solids (2:1)
AHEA F-41	Al(OH) <sub>3</sub> -Egg albumin (4:1)
AHLA F-41	Al(OH) <sub>3</sub> -Lactalbumin (4:1)
AHGEL F-41	Al(OH) <sub>3</sub> -Gelatin (4:1)
AHMPC F-41	Al(OH) <sub>3</sub> -Milk protein concentrate (4:1)
AHS F-41	Al(OH) <sub>3</sub> -Soya milk (4:1)
AHWM F-11	Al(OH) <sub>3</sub> -Whole milk solids (1:1)

<sup>a</sup> Registered trademark, Reheis Co., Inc.

<sup>b</sup> Al(OH)<sub>3</sub> as Al<sub>2</sub>O<sub>3</sub>.

**Antacid Activity.**—The antacid activity of the various samples of aluminum hydroxide-protein dried gels was determined by the modified procedure of Holbert, Noble, and Grote (2) on the basis of 1-2-Gm. doses. In addition, the antacid activity of 10-grain aluminum hydroxide-non-fat milk tablets (ALUMIL F-21) was determined on the basis of one and two tablets. The results of these determinations are shown in Table III and Figs. 1-8.

**Effect of Nonfat Milk Solids on Antacid Activity of Aluminum Hydroxide Dried Gel.**—In order to determine the effect of nonfat milk solids on the antacid activity of aluminum hydroxide dried gel, two parts of the latter were blended thoroughly with one part of nonfat milk solids (Carnation Instant). The antacid activity of a 3-Gm. sample was determined by the modified procedure of Holbert, Noble, and Grote and compared with a 2-Gm. sample of dried aluminum hydroxide gel. The data which are plotted in Fig. 9 show that nonfat milk solids have an adverse effect on the antacid properties of aluminum hydroxide dried gel. Similar results were obtained with whole milk solids (Klim.). A 2-Gm. sample of nonfat milk solids (Carnation Instant), similarly tested, raised the pH slowly to 2.4 and gradually declined to less than 2.0. A 1-Gm. mixture of 38 parts malted milk with 10 parts aluminum hydroxide dried gel (4) failed to raise the pH of gastric juice above 2.0 during the entire two-hour testing period.

One gram of a Lipschitz (3) mixture, previously described, raised the pH fairly rapidly to 3.6, and

\* Received September 2, 1959, from the Research Laboratory, Reheis Co., Inc., Berkeley Heights, N. J.  
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.



TABLE II.—ANALYSES OF VARIOUS ALUMINUM HYDROXIDE-PROTEIN DRIED GELS

Designation	Al <sub>2</sub> O <sub>3</sub> , %	Protein, %	Nonfat Milk Solids, %	CO <sub>2</sub> , %	Water, Karl Fischer, %	pH	Acid Consuming Cap. <sup>a</sup>
ALUMIL F-21	38.0	7.0	19.0	12.1	18.8	9.0	220
AHEA F-41	46.1	11.4	..	9.5	16.6	9.1	257
AHLA F-41	46.0	9.2	..	12.5	22.1	9.0	256
ANGEL F-41	42.5	10.5	..	11.2	17.0	9.1	234
AHMPC F-41	42.2	6.8	..	12.7	18.6	9.1	231
AHS F-41	42.4	2.9	..	15.0	22.4	9.3	232
AHWM F-11	31.7	8.7	..	8.9	20.1	9.1	179

a Ml. 0.1 N HCl per Gm.

TABLE III.—ANTACID CHARACTERISTICS OF VARIOUS ALUMINUM HYDROXIDE-PROTEIN DRIED GELS<sup>a</sup>

Time, min.	ALUMIL F-21,		AHEA F-41,		AHLA F-41,		ANGEL F-41,		AHMPC F-41,		AHS F-41,		AHWM F-11,		ALUMIL Tablets,	
	1 Gm.	2 Gm.	1 Gm.	2 Gm.	1 Gm.	2 Gm.	1 Gm.	2 Gm.	1 Gm.	2 Gm.	1 Gm.	2 Gm.	1 Gm.	2 Gm.	1 Tab.	2 Tab.
0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
1	3.7	4.1	2.5	4.1	2.5	4.1	2.8	4.1	3.6	4.2	2.5	4.2	3.3	4.1	2.2	2.8
2	4.1	4.2	3.2	4.2	3.0	4.2	3.8	4.2	4.1	4.3	3.2	4.3	3.8	4.2	2.6	3.5
3	4.2	4.2	3.9	4.2	3.5	4.3	4.0	4.2	4.2	4.3	3.8	4.3	4.0	4.2	3.1	3.9
4	4.2	4.2	4.1	4.2	3.9	4.3	4.0	4.2	4.2	4.3	4.0	4.3	4.0	4.2	3.5	4.0
5	4.2	4.2	4.1	4.2	4.0	4.3	4.0	4.2	4.2	4.4	4.1	4.4	4.1	4.2	3.7	4.1
10	4.3	4.3	4.3	4.3	4.2	4.4	4.1	4.2	4.2	4.4	4.1	4.4	4.2	4.2	4.1	4.2
20	4.3	4.3	4.2	4.3	4.2	4.4	4.1	4.2	4.2	4.4	4.1	4.4	4.1	4.2	4.1	4.2
30	4.3	4.2	4.2	4.3	4.2	4.4	4.1	4.2	4.2	4.4	4.1	4.3	4.0	4.1	4.1	4.2
40	4.3	4.2	4.2	4.3	4.2	4.4	4.1	4.2	4.2	4.4	4.1	4.3	4.0	4.1	4.1	4.2
50	4.3	4.2	4.2	4.2	4.2	4.4	4.1	4.1	4.2	4.4	4.1	4.3	4.0	4.1	4.0	4.1
60	4.3	4.2	4.2	4.2	4.2	4.4	4.1	4.1	4.2	4.3	4.1	4.3	3.9	4.1	4.0	4.1
70	4.3	4.2	4.2	4.2	4.1	4.3	4.0	4.1	4.2	4.3	4.0	4.3	3.8	4.0	3.9	4.1
80	4.2	4.2	4.1	4.2	4.1	4.3	4.0	4.1	4.1	4.3	4.0	4.3	3.8	4.0	3.8	4.0
90	4.2	4.2	4.1	4.2	4.1	4.3	4.0	4.1	4.1	4.3	3.9	4.3	3.6	4.0	3.6	4.0
100	4.2	4.2	4.1	4.2	4.0	4.2	3.9	4.1	4.0	4.3	3.7	4.3	3.4	3.9	3.4	3.9
110	4.1	4.2	4.1	4.2	4.0	4.2	3.8	4.1	4.0	4.2	3.3	4.3	3.2	3.9	3.2	3.7
120	4.1	4.2	4.0	4.2	3.9	4.2	3.7	4.1	3.7	4.2	2.9	4.2	2.9	3.8	2.9	3.6
130	4.0	4.1	4.0	4.2	3.8	4.2	3.3	4.0	3.3	4.2	..	4.2	..	3.8	..	3.3
140	3.8	4.1	3.8	4.1	3.5	4.2	3.0	4.0	3.0	4.1	..	4.2	..	3.6	..	..
150	3.5	4.0	3.7	4.1	..	4.2	..	4.0	2.7	4.0	..	4.1	..	3.4	..	..
160	3.2	4.0	3.3	4.1	..	4.1	..	3.9	2.6	4.0	..	4.1	..	3.2	..	..
170	3.0	3.7	3.0	4.1	..	4.0	..	3.9	2.4	3.9	..	3.9	..	2.9	..	..
180	..	3.5	..	4.0	..	3.8	..	3.8	2.3	3.4	..	3.6	..	2.7	..	..
190	..	3.3	..	3.9	..	3.3	..	3.6	..	3.0	..	3.1	..	..	..	..
200	..	2.9	..	3.7	..	3.0	..	3.3	..	2.8	..	2.8	..	..	..	..
210	..	..	..	3.5	..	2.8	..	3.0	..	2.6	..	2.6	..	..	..	..

a Time versus pH at various dose levels, procedure of Holbert, Noble, and Grote modified.

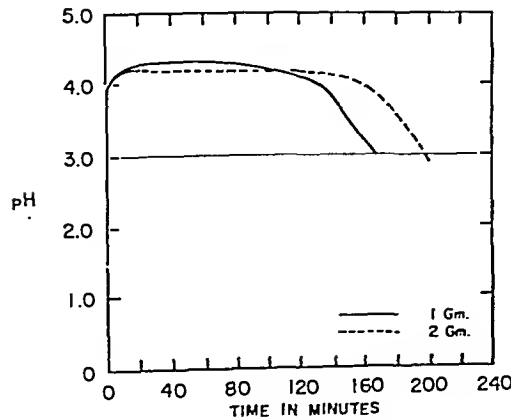


Fig. 1.—Aluminum hydroxide-nonfat milk dried gel, ALUMIL F-21, procedure of Holbert, Noble, and Grote modified.

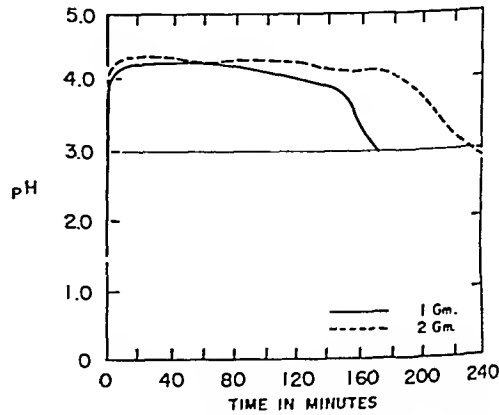


Fig. 2.—Aluminum hydroxide-egg albumin dried gel, AHEA F-41, procedure of Holbert, Noble, and Grote modified.

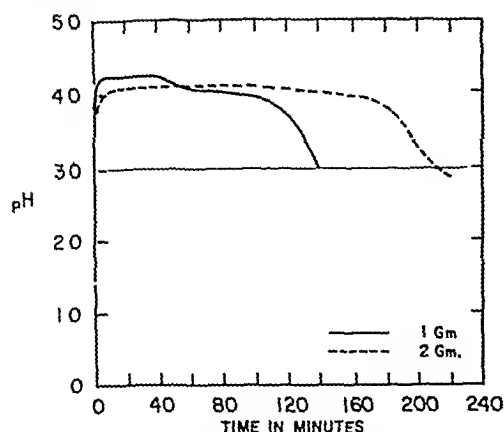


Fig. 3—Aluminum hydroxide-gelatin dried gel, AHGEL F-41, procedure of Holbert, Noble, and Grote modified.

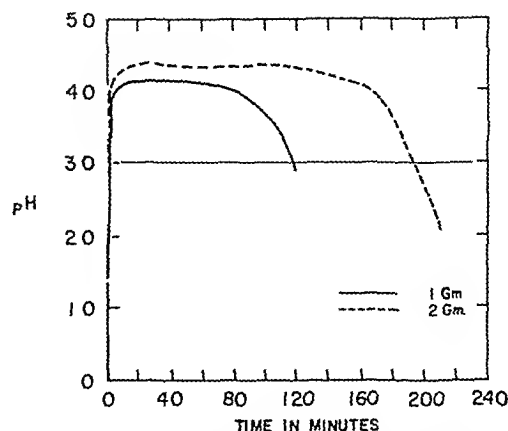


Fig. 6—Aluminum hydroxide-soya dried gel, AHS F-41, procedure of Holbert, Noble, and Grote modified.

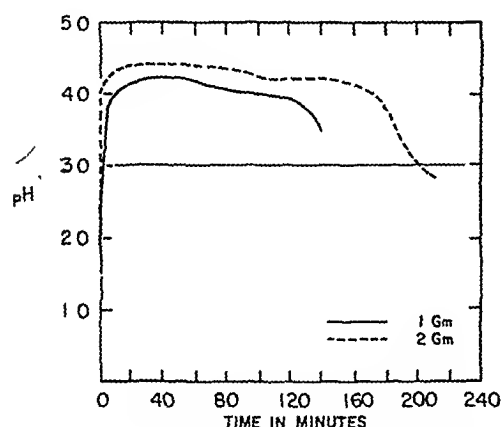


Fig. 4—Aluminum hydroxide-lactalbumin dried gel, AH-LA F-41, procedure of Holbert, Noble, and Grote modified.

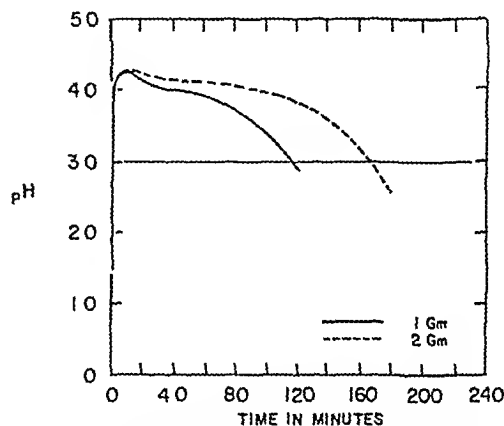


Fig. 7—Aluminum hydroxide-whole milk dried gel, AHWM F-11, procedure of Holbert, Noble, and Grote modified.

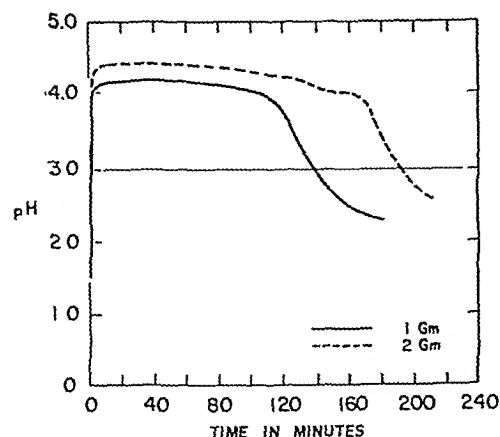


Fig. 5—Aluminum hydroxide-milk protein concentrate dried gel, AHMPC F-41, procedure of Holbert, Noble, and Grote modified.

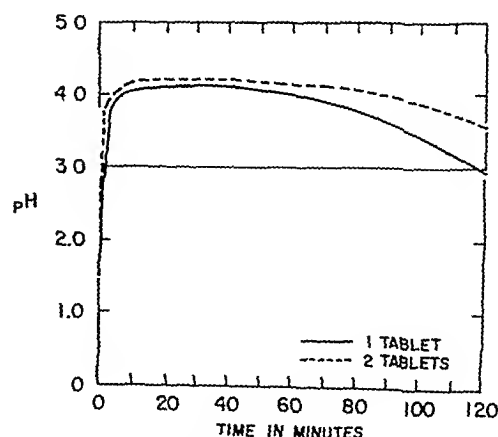


Fig. 8—Alumil F-21 tablets, 660 mg. active ingredient, procedure of Holbert, Noble, and Grote modified.

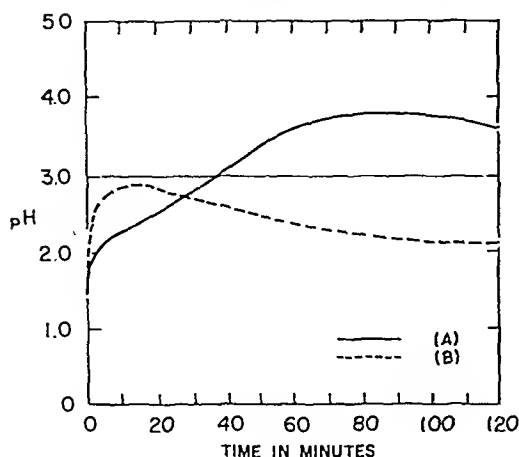


Fig. 9.—Effect of dry nonfat milk solids on antacid activity of aluminum hydroxide dried gel U. S. P.: (A) Aluminum hydroxide dried gel, 2 Gm., (B) Aluminum hydroxide dried gel, 2 Gm. plus 1 Gm. nonfat milk solids; procedure of Holbert, Noble, and Grote modified.

thereafter, slowly declined to 3.0 in twenty minutes and 2.0 after one hundred minutes. The antacid characteristics of aluminum proteinate (5) are shown in Fig. 9 and in Table V of a previous paper (1).

### RESULTS

The data on the antacid activity of various aluminum hydroxide-protein dried gels, which are shown in Table III and Figs. 1-8, indicate they are prompt in raising the pH to 3.5 and very prolonged in maintaining the pH in the optimum range of 3 to 5. The maximum pH reached with a large excess of antacid was about 4.4. One-gram samples maintained the pH in the optimum range for well over two hours and 2-Gm. samples for well over three hours. This is in sharp contrast with the data shown in Fig. 9, which show that nonfat milk solids have an adverse effect on the antacid activity of aluminum hydroxide dried gel. The data plotted in Fig. 8 show that the prompt and prolonged antacid activity of ALUMIL F-21 is retained in tablets as well.

### DISCUSSION

The antacid properties of aluminum hydroxide-protein dried gels, as determined by the modified *in*

*vitro* technique of Holbert, Noble, and Grote, have been shown to compare favorably with reactive liquid aluminum hydroxide gel. The results obtained with aluminum hydroxide-nonfat milk (ALUMIL), which is a most economical source of protein, were comparable with others from the point of view of promptness and long lasting buffering power in the optimum pH range. Tablets made from ALUMIL were characterized by a smooth, bland, nonchalky taste and unexcelled sucking quality. A vanillin flavor gave a pleasant taste and aftertaste. Favorable comments were elicited from individuals who dislike "chalky" or highly flavored tablets.

The prolonged buffering quality of ALUMIL suggests its use for night-time therapy of ulcer patients. Other antacid chemicals, such as magnesium hydroxide, can be most effectively incorporated by blending with the wet gel before drying.

### SUMMARY

1. The preparation and properties of seven new aluminum hydroxide-protein dried gels were described.

2. Each was shown to exhibit promptness in buffering artificial gastric juice and prolonged antacid action in the optimum pH range of 3 to 5 when evaluated by the modified *in vitro* procedure of Holbert, Noble, and Grote.

3. The antacid properties were shown to compare favorably with reactive liquid aluminum hydroxide gel.

4. The antacid activity of ALUMIL F-21 (aluminum hydroxide-nonfat milk dried gel) tablets was shown to be prompt and prolonged.

5. The aesthetic qualities of aluminum hydroxide-nonfat milk dried gel (ALUMIL) such as the bland, nonchalky, pleasant taste characteristics as well as the smooth, nongritty, sucking qualities which it imparts to tablets have been stressed.

### REFERENCES

- (1) Beckman, S. M., *THIS JOURNAL*, 49, 191 (1960).
- (2) Holbert, J. M., Noble, N., and Grote, I. W., *Ibid.*, 37, 292 (1948).
- (3) U. S. pat. 2,362,386.
- (4) British pat. 715,874.
- (5) U. S. pat. 2,721,861.

# The Effects of Certain Psychopharmacologic Drugs on Conditioning in the Rat I\*

## Avoidance-Escape Conditioning

By V. D. LYNCH†, M. D. ACETO‡, and R. K. THOMS

This study compares the effects on conditioning of well-known classes of tranquilizers both with themselves and with other centrally active drugs. On the basis of certain chemical structure relationships, 2-ethyl-2-(3-methyl)-butylmalondiamide, 2-ethyl-2-(3-methyl)-butylmalonic acid diethylester, and ethyl-2,4-toluene dicarbamate were included for study. The results which were analyzed statistically by means of an analysis of variance technique and *t* tests demonstrated significant differences between drugs and between drugs and controls from both dosage and trial aspects. Because of the nature of the test, these differences cannot be ascribed only to an interference with normal motor activity.

WHILE MANY PROCEDURES have been used for the assessment of tranquilizers, the experimental approach has logically focused on the effects of these drugs on animal behavior (1, 2). In an attempt to contribute somewhat to a further understanding of such phenomena, the following study was carried out, keeping in mind the threefold objectives (a) to determine whether quantitative relationships could be determined not only between drugs but also between different dosage levels of the same drugs; (b) to compare the effects of different chemical classes of drugs; and (c) to determine whether or not this procedure could be used to evaluate new or untested chemical compounds.

In the study of centrally active drugs on animal behavior, Cook and Weidley (3) utilized a procedure in which a rat was conditioned to climb a pole to escape an electric shock which was delivered simultaneously with a warning tone. After a number of such presentations of shock and tone together, the animal would develop a response termed avoidance-escape by the authors and it would respond to the sound alone.

In the present study, the same procedure was utilized but modified to the extent that a time interval was introduced between the warning tone and the electric shock. In addition, other parameters were extended with regard to the

time of administration of drugs. It was also felt that the administration of a drug to an unconditioned animal rather than to one already conditioned would more fully elucidate the effects of the selected drugs.

### EXPERIMENTAL PROCEDURES

**Materials.**—The drugs and dosage levels are listed in Table I. Except for the diamide<sup>1</sup> and dicarbamate (4) which were synthesized in our laboratories, all the other drugs were obtained commercially or supplied by the manufacturers.<sup>2</sup> Distilled water was used as the solvent for the relatively water-soluble drugs. Methocel (0.5%) was used as the suspending agent for the poorly water-soluble drugs. In all cases, the drug was either dissolved or suspended so that the desired dose per Kg. of body weight of the rat was contained in a final volume of 1 cc of water. When not in use, the solutions were stored in a refrigerator.

**Animals.**—The animals used in this study were descendants of a Wistar strain originally obtained from the Charles River Breeding Laboratories. Adult rats were kept in colonies of approximately 25 to a standard size rat cage. Separate cages were maintained for both sexes. These animals were allowed Purina laboratory chow for food and water *ad libitum*. Once selected for use in the avoidance-escape procedure, the animals were individually housed in rectangular mouse boxes, and food and water allowed *ad libitum*.

**Conditioning Apparatus.**—The apparatus consisted of a cage (both wooden and glass containers were used in these experiments) fitted with a cover to which a wooden rod was attached by means of a flat metal spring. This rod protruded through the center of the cover and extended down almost to the floor of the cage. The weight of an animal on this

\* Received May 26, 1959, from the School of Pharmacy, University of Connecticut, Storrs.

† Abstracted in part from dissertations submitted to the Graduate School by Vincent D. Lynch and Mario D. Aceto in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June 1959.

‡ Present address: College of Pharmacy, St. John's University, Jamaica 32, N. Y.

§ Present address: School of Pharmacy, University of Pittsburgh, Pittsburgh 13, Pa.

The authors wish to thank Dr. David Zeman of the Psychology Department of the University of Connecticut and Dr. Robert Orlando of the Institute for Child Development of the University of Washington for their valuable assistance in developing the psychological procedures and the statistical analysis of the data.

<sup>1</sup> The synthesis and pharmacological data on this compound to be published.

<sup>2</sup> The following drugs were supplied gratis from the firms listed: 2-ethyl-2-(3-methyl)-butylmalonic acid diethyl ester from Eli Lilly and Co.; Equanil (meprobamate) from Wyeth Laboratories; Thorazine hydrochloride from Smith Kline and French Laboratories; Atarax hydrochloride from J. B. Roerig and Co. (Div. of Charles Pfizer & Co., Inc.); Serpasil phosphate (lyophilized crystals) from Ciba Pharmaceutical Products Inc.

TABLE I.—DRUGS AND DOSES USED IN AVOIDANCE-ESCAPE CONDITIONING TECHNIQUE<sup>a</sup>

Drug	Lot and Control No.	Low, mg./Kg.	Medium, mg./Kg.	High, mg./Kg.
Reserpine phosphate	Ciba E 4961	0.5	1.0	2.0
Chlorpromazine hydrochloride	SKF Cu 7664	0.5	2.0	4.0
Meprobamate	Wyeth L 3286-1	10.0	50.0	100.0
Hydroxyzine hydrochloride	Pfizer 71407	5.0	25.0	35.0
<i>d</i> -Amphetamine sulfate	Lack DA 675	0.1	1.0	5.0
Amobarbital	Eli Lilly AX-9643-F	5.0	10.0	20.0
Ethyl-2,4-toluene dicarbamate	....	50.0	75.0	90.0
2-Ethyl-2-(3-methyl)-butylmalondiamide	....	10.0	50.0	100.0
2-Ethyl-2-(3-methyl)-butylmalonic acid diethyl ester	Eli Lilly 11039	50.0	100.0	200.0
Control	....	..	..	..

<sup>a</sup> All drugs given by i. p. injection.

pole would cause the pole to descend, thereby engaging electrical contacts at the top of the pole which immediately terminated the warning sound and/or electric shock. The cage floor was constructed as a grid of copper rods spaced approximately one-half inch apart. The grid floor could be electrically charged by means of a stimulator. The stimulator (Electronic Stimulator, Grass Instrument Co.) frequency was set and maintained at five per second, delay at five milliseconds, duration at thirty milliseconds, and voltage at 100 volts d. c. A resistor was so set that three milliamperes current was delivered to the grid.

The apparatus was constructed so that approximately every five minutes it would initiate a new cycle which began by the sounding of a low tone buzzer for approximately sixteen seconds followed by the delivery of an electric shock and continued sounding of the buzzer (with concurrent shock) until either the animal jumped upon the pole or, as was found necessary for certain drugs during the first hourly session, until the animal became cyanotic. If the animal became cyanotic, the investigator manually depressed the protruding end of the pole. Either manual depression of the pole or the presence of the animal upon the pole served to terminate simultaneously the buzzer tone and electric shock. The apparatus would automatically reset itself and after approximately five minutes initiate a new cycle unless the rat was still on the pole, in which case another cycle would begin approximately five minutes after the animal left the pole. A kymograph (Phipps and Bird, model V.H.) separately recorded duration of the tone and shock by means of ink-fed pens attached to signal magnets.

The animal was introduced into the apparatus by raising the removable roof. Two of these devices were used and each apparatus was housed in a separate room in an exhaust hood.

**Procedure.**—In each of the apparatuses, five rats were used to form a group for each dose level investigated for each drug. Once a group was assigned to an apparatus, it was used only in the apparatus assigned. The similar results obtained for each dose level for each drug from each apparatus were then combined to permit the best possible statistical analysis. Experimentally naïve animals of both sexes of a weight range from 150 to 250 Gm. were used. Aside from the control groups which did not receive any injections, all other groups were injected intraperitoneally one hour prior to each of the five consecutive daily admissions into the apparatus. Reserpine was administered two hours prior and

meprobamate one-half hour prior to admission of the animal into the apparatus. Due to the cumulative effects of reserpine, it was found necessary to administer this drug and conduct the conditioning session every other day for a total of three sessions.

After injecting an animal with the selected drug it was replaced in its assigned cage and remained there until ready for introduction into the apparatus. The animal was removed from the cage and placed in tap water of sufficient depth to allow wetting of its limbs and then admitted into the apparatus. After the first few shocks the animal learned to find the pole and responded in time according to the effects of the drug. After a conditioning session, each rat was returned to its assigned cage.

RESULTS

In the evaluation of the data, the original measurements on the kymograph record were made in millimeters and then converted to response latency in seconds. Response latency may be described as the time interval which elapses between the onset of the warning tone and response of the animal, in this case, climbing the pole to avoid an oncoming shock. Each animal received an average of ten trials in each daily session, and because the results of these trials showed skewness, the measurement for each trial was converted to its reciprocal.

The average of these reciprocals ( $\times 100$ ) for each animal on each day was entered into a table and analyzed statistically by means of a split-plot analysis of variance technique (5). The results of this technique are based on the measurements of approximately 15,000 individual trials and are given in Table II.

Using the *t* test<sup>3</sup> as defined by Edwards (6), a further statistical evaluation of the various drug to drug and drug to control comparisons was made. Those results are listed in Tables III, IV, and V. Figures 1 through 9 illustrate the trial-latency curves for the different drugs and doses investigated. Table VI provides a summary and tabulation of the drugs and dose levels at which significant results were obtained.

3

TABLE OF <i>t</i> VALUES (6)		
<i>df</i>	0.05	0.01
18	2.101	2.878

TABLE II.—SPLIT-PLOT ANALYSIS OF VARIANCE OF THE EFFECTS OF NINE DRUGS ON THE ACQUISITION OF CONDITIONING OF WHITE RATS IN AN AVOIDANCE-ESCAPE SITUATION USING RESPONSE LATENCY AS THE MEASURE<sup>a</sup>

Source of Variation	Sum of Squares	df	Mean Square	F	F 1% (Fisher's Tables)
<b>Main plot</b>					
A-Drugs	171,680.41	8	21,460.05	15.39	2.60
B-Levels	56,580.05	2	28,290.02	20.09	4.71
A × B Interaction	118,500.07	16	7,406.25	5.31	2.09
Main plot error	338,667.23	243	1,393.69	..	..
<b>Subplot</b>					
T-Trials	86,537.71	4	21,634.42	56.38	3.32
A × T Interaction	27,030.02	32	844.69	2.20	1.69
B × T Interaction	5,023.20	8	627.90	1.64	2.51
A × B × T Interaction	52,481.45	64	820.02	2.14	1.54
Subplot error	337,003.60	972	383.75	..	..
Total	1,193,503.74	1,349	...	..	..

<sup>a</sup> Each drug tested at three different dosage levels.

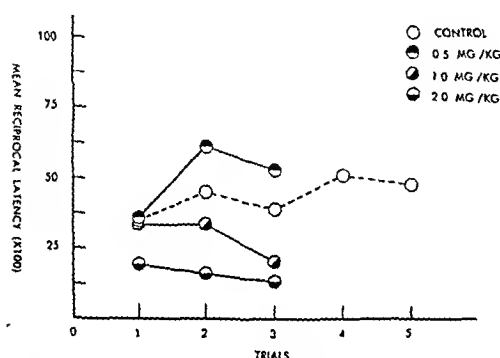


Fig. 1.—The effect of reserpine  $PO_4$  on avoidance-escape.

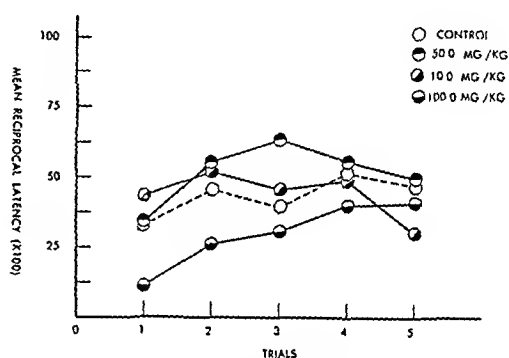


Fig. 3.—The effect of meprobramate on avoidance-escape.

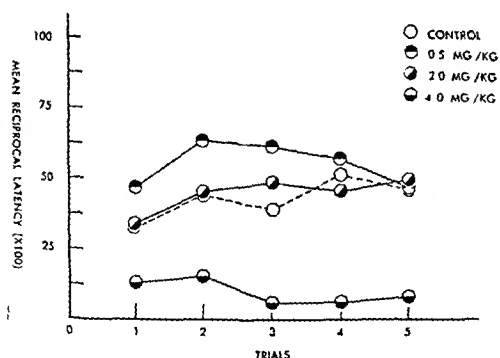


Fig. 2.—The effect of chlorpromazine HCl on avoidance-escape.

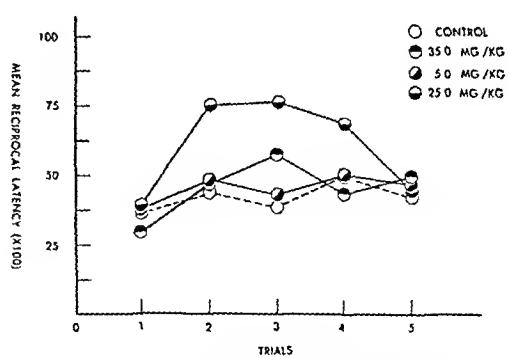


Fig. 4.—The effect of hydroxyzine HCl on avoidance-escape.

## DISCUSSION AND SUMMARY

The F values obtained by means of a split-plot analysis of variance of the data indicate that many significant drug to drug differences exist considered from both dosage and trial aspects. The *t* tests derived from these data lend further support and indicate the specific drug to drug significant differences and the drug to control significant differences.

On the assumption of the validity of the significance of the F ratios for both the trial and trial-drug interactions, an inspection of the different curves in Figs 1 through 9 shows that the differences generally arise on the second trial, and that generally these differences are maintained throughout the remaining three trials.

Reserpine was effective in interfering with the avoidance-escape response significantly in a dose of one to two mg./Kg. The data, as presented, does

TABLE III.—TABLE OF *t* VALUES DERIVED FROM VARIOUS DRUG TO DRUG AND DRUG TO CONTROL COMPARISONS, LOW DOSAGE LEVEL

Drug	1	2	3	4	5	6	7	8	9	10
1 Reserpine phosphate	..	1.80	1.34	0.88	0.17	0.13	1.70	2.78	1.49	1.08
2 Chlorpromazine hydrochloride	.	.	1.29	0.89	0.28	0.77	1.24	1.93	0.96	1.04
3 Meprobamate	.	.	..	0.64	1.48	2.48	3.15	5.09	3.41	0.20
4 Hydroxyzine hydrochloride	.	.	..	..	1.05	2.19	2.85	4.04	3.14	0.58
5 <i>d</i> -Amphetamine sulfate	.	.	..	..	..	0.93	1.48	2.44	1.22	1.23
6 Amobarbital	.	.	..	..	..	..	0.57	1.35	0.09	2.22
7 Ethyl-2,4-toluene dicarbamate	.	.	..	..	..	..	..	..	..	2.78
8 2-Ethyl-2-(3-methyl)-butyl malon-diamide	.	.	..	..	..	..	..	..	1.89	4.23
9 2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester	.	.	..	..	..	..	..	..	..	2.86
10 Control	..	..	..	..	..	..	..	..	..	..
df = 18										

TABLE IV.—TABLE OF *t* VALUES DERIVED FROM VARIOUS DRUG TO DRUG AND DRUG TO CONTROL COMPARISONS, MEDIUM DOSAGE LEVEL

Drug	1	2	3	4	5	6	7	8	9	10
1 Reserpine phosphate	.	2.64	3.42	6.60	6.68	6.07	6.23	2.17	5.66	3.17
2 Chlorpromazine hydrochloride	.	.	0.68	2.05	2.72	2.20	2.28	0.81	1.70	0.11
3 Meprobamate	.	..	..	1.20	1.94	1.21	1.50	1.57	0.89	0.89
4 Hydroxyzine hydrochloride	.	.	..	..	1.09	0.09	0.47	3.58	0.35	2.61
5 <i>d</i> -Amphetamine sulfate	.	.	..	..	..	0.95	0.59	4.12	1.33	3.32
6 Amobarbital	.	.	..	..	..	..	0.36	0.34	0.40	2.61
7 Ethyl-2,4-toluene dicarbamate	.	.	..	..	..	..	..	..	..	2.83
8 2-Ethyl-2-(3-methyl)-butyl malon-diamide	..	.	..	..	..	..	..	..	3.02	0.83
9 2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester	.	.	..	..	..	..	..	..	..	2.17
10 Control	..	..	..	..	..	..	..	..	..	..
df = 18										

TABLE V.—TABLE OF *t* VALUES DERIVED FROM VARIOUS DRUG TO DRUG AND DRUG TO CONTROL COMPARISONS, HIGH DOSAGE LEVEL

Drug	1	2	3	4	5	6	7	8	9	10
1 Reserpine phosphate	.	0.76	3.76	4.55	4.70	4.78	10.96	7.24	5.59	4.51
2 Chlorpromazine hydrochloride	.	..	5.73	5.84	5.63	6.96	14.29	9.08	7.79	5.75
3 Meprobamate	.	.	..	2.09	2.48	1.74	8.14	4.45	2.82	1.56
4 Hydroxyzine hydrochloride	.	.	..	..	0.32	0.94	3.47	1.37	0.03	0.68
5 <i>d</i> -Amphetamine sulfate	.	.	..	..	..	1.28	2.91	0.95	0.34	1.00
6 Amobarbital	.	.	..	..	..	..	5.93	2.87	1.24	0.21
7 Ethyl-2,4-toluene dicarbamate	.	.	..	..	..	..	..	..	..	4.92
8 2-Ethyl-2-(3-methyl)-butyl malondiamide	.	.	..	..	..	..	..	..	1.66	2.31
9 2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester	.	.	..	..	..	..	..	..	..	0.89
10 Control	.	..	..	..	..	..	..	..	..	..
df = 18										

TABLE VI.—DRUGS SHOWING A SIGNIFICANT EFFECT ON RESPONSE TIME WHEN COMPARED TO CONTROL (*P* = 0.05)

Drug	Effect	Dose, mg./Kg	No response effect at following doses, mg./Kg
Reserpine phosphate	Inhibited response	1.0, 2.0	0.5
Chlorpromazine hydrochloride	Inhibited response	4.0	0.5, 2.0
Hydroxyzine hydrochloride	Facilitated response	25.0	5.0, 35.0
<i>d</i> -Amphetamine sulfate	Facilitated response	1.0	0.1, 5.0
Amobarbital	Facilitated response	5.0, 10.0	20.0
Ethyl-2,4-toluene dicarbamate	Facilitated response	50.0, 75.0, 90.0	.....
2-Ethyl-2-(3-methyl)-butyl malondiamide	Facilitated response	10.0, 100.0	50.0
2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester	Facilitated response	50.0, 100.0	200.0
Meprobamate	.....	.....	10.0, 50.0, 100.0

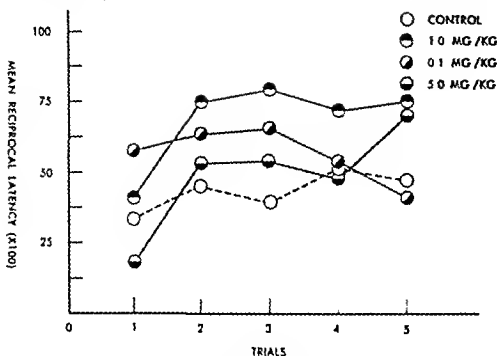


Fig. 5.—The effect of *d*-amphetamine  $\text{SO}_4$  on avoidance-escape.

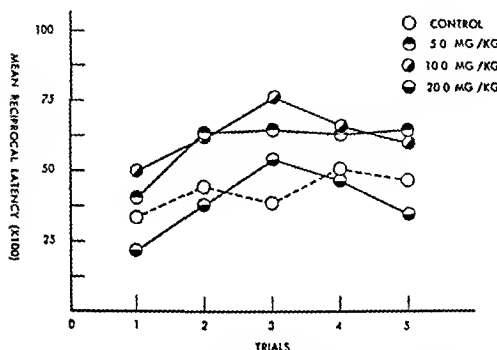


Fig. 6.—The effect of amobarbital on avoidance-escape.

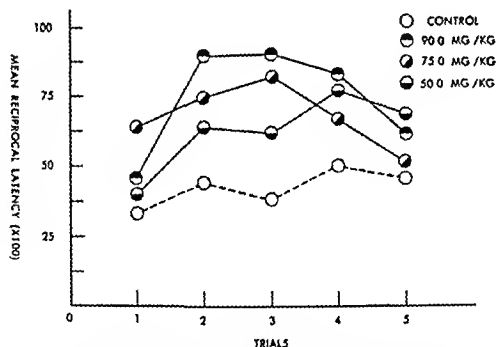


Fig. 7.—The effect of ethyl-2,4-toluene dicarbamate on avoidance-escape.

not justify a conclusion as to whether this drug interfered with the acquisition of conditioning, or whether it prevented the animal from responding. Diarrhea, and ptosis of the eyelids, in addition to sedation, were obvious in all animals receiving this drug by the third trial (in this case five days after the first trial). The highest dose of reserpine was the first to manifest these results. The activity of reserpine phosphate was found to be cumulative.

Chlorpromazine hydrochloride was found to have a significant effect on conditioning in a four mg./Kg. dose. The curve for this drug indicates that at this dose chlorpromazine appears to interfere with the

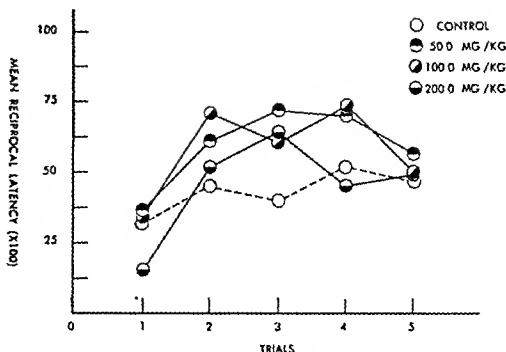


Fig. 8.—The effect of 2-ethyl-2-(3-methyl)-butyl malonic acid diethyl ester on avoidance-escape.

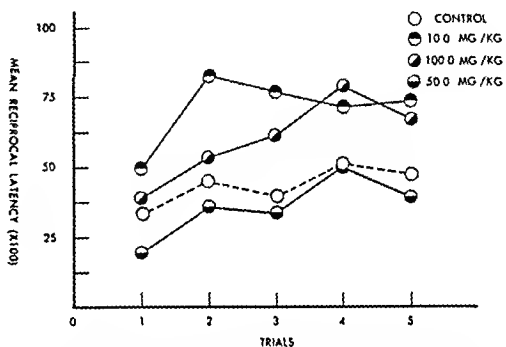


Fig. 9.—The effect of 2-ethyl-2-(3-methyl)-butylmalondiamide on avoidance-escape.

avoidance response to a greater extent than with the escape component. However, the response at this dose level is not significantly different from that for reserpine at the highest dose of the latter.

According to the *t* tests, meprobamate does not interfere significantly with conditioning at any dose level. An examination of the response curves indicates an increase in response latency for the highest dose, especially on trial days two and four. This might be consistent with the observation of pronounced flaccid paralysis in all the rats studied at this dose.

The effects of hydroxyzine hydrochloride on avoidance-escape conditioning as reflected by the *t* tests indicate a decrease in response latency at a dose of 25 mg./Kg., whereas no significant effect occurred at a dose of 35 mg./Kg. It is proposed that the highest dose used was beginning to interfere with its own activity at this point, since doses higher than 35 mg./Kg. could not be investigated because such doses caused pronounced convulsive seizures.

It is possible that amphetamine sulfate followed the same pattern of activity since similar results were obtained with this drug, i. e., the median dose facilitating the response and the stimulatory effects of the highest dose interfering with this facilitation.

On the other hand, amobarbital significantly decreased response latency at the low and median dose levels when compared to the controls. This would be consistent with the observation that barbiturates



TABLE III.—TABLE OF *t* VALUES DERIVED FROM VARIOUS DRUG TO DRUG AND DRUG TO CONTROL COMPARISONS, LOW DOSAGE LEVEL

Drug	1	2	3	4	5	6	7	8	9	10
1 Reserpine phosphate		1.80	1.34	0.88	0.17	0.13	1.70	2.78	1.49	1.00
2 Chlorpromazine hydrochloride			1.29	0.89	0.28	0.77	1.24	1.93	0.96	1.00
3 Meprobamate				0.64	1.48	2.48	3.15	5.09	3.41	0.20
4 Hydroxyzine hydrochloride					1.05	2.19	2.85	4.04	3.14	0.30
5 <i>d</i> -Amphetamine sulfate						0.93	1.48	2.44	1.22	1.20
6 Amobarbital							0.57	1.35	0.09	2.20
7 Ethyl-2,4-toluene dicarbamate										2.70
8 2-Ethyl-2-(3-methyl)-butyl malon-diamide									1.89	4.20
9 2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester										2.80
10 Control										

df = 18

TABLE IV.—TABLE OF *t* VALUES DERIVED FROM VARIOUS DRUG TO DRUG AND DRUG TO CONTROL COMPARISONS, MEDIUM DOSAGE LEVEL

Drug	1	2	3	4	5	6	7	8	9	10
1 Reserpine phosphate		2.64	3.42	6.60	6.68	6.07	6.23	2.17	5.66	3.17
2 Chlorpromazine hydrochloride			0.68	2.05	2.72	2.20	2.28	0.81	1.70	0.11
3 Meprobamate				1.20	1.94	1.21	1.50	1.57	0.89	0.89
4 Hydroxyzine hydrochloride					1.09	0.09	0.47	3.58	0.35	2.64
5 <i>d</i> -Amphetamine sulfate						0.95	0.59	4.12	1.33	3.32
6 Amobarbital							0.36	0.34	0.40	2.61
7 Ethyl-2,4-toluene dicarbamate										2.83
8 2-Ethyl-2-(3-methyl)-butyl malon-diamide									3.02	0.83
9 2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester										2.17
10 Control										

df = 18

TABLE V.—TABLE OF *t* VALUES DERIVED FROM VARIOUS DRUG TO DRUG AND DRUG TO CONTROL COMPARISONS, HIGH DOSAGE LEVEL

Drug	1	2	3	4	5	6	7	8	9	10
1 Reserpine phosphate		0.76	3.76	4.55	4.70	4.78	10.96	7.24	5.59	4.51
2 Chlorpromazine hydrochloride			5.73	5.84	5.63	6.96	14.29	9.08	7.79	5.75
3 Meprobamate				2.09	2.48	1.74	8.14	4.45	2.82	1.59
4 Hydroxyzine hydrochloride					0.32	0.94	3.47	1.37	0.03	0.68
5 <i>d</i> -Amphetamine sulfate						1.28	2.91	0.95	0.34	1.00
6 Amobarbital							5.93	2.87	1.24	0.24
7 Ethyl-2,4-toluene dicarbamate										4.92
8 2-Ethyl-2-(3-methyl)-butyl malondiamide									1.66	2.34
9 2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester										0.89
10 Control										

df = 18

TABLE VI.—DRUGS SHOWING A SIGNIFICANT EFFECT ON RESPONSE TIME WHEN COMPARED TO CONTROL ( $P \approx 0.05$ )

Drug	Effect	Dose, mg /Kg	No response effect at following doses, mg /Kg.
Reserpine phosphate	Inhibited response	1.0, 2.0	0.5
Chlorpromazine hydrochloride	Inhibited response	4.0	0.5, 2.0
Hydroxyzine hydrochloride	Facilitated response	25.0	5.0, 35.0
<i>d</i> -Amphetamine sulfate	Facilitated response	1.0	0.1, 5.0
Amobarbital	Facilitated response	5.0, 10.0	20.0
Ethyl-2,4-toluene dicarbamate	Facilitated response	50.0, 75.0, 90.0	...
2-Ethyl-2-(3-methyl)-butyl malondiamide	Facilitated response	10.0, 100.0	50.0
2-Ethyl-2-(3-methyl)-butyl malonic acid diethyl ester	Facilitated response	50.0, 100.0	200.0
Meprobamate	.....	.....	10.0, 50.0, 100.0

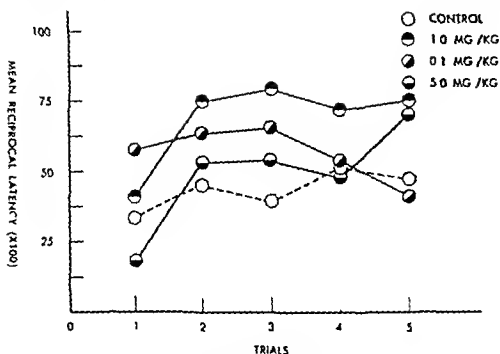


Fig. 5.—The effect of *d*-amphetamine  $\text{SO}_4$  on avoidance-escape.

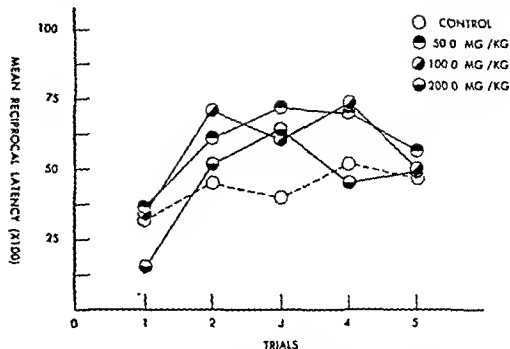


Fig. 8.—The effect of 2-ethyl-2-(3-methyl-butyl) malonic acid diethyl ester on avoidance-escape.

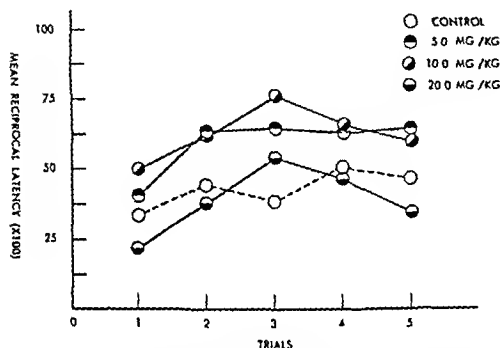


Fig. 6.—The effect of amobarbital on avoidance-escape.

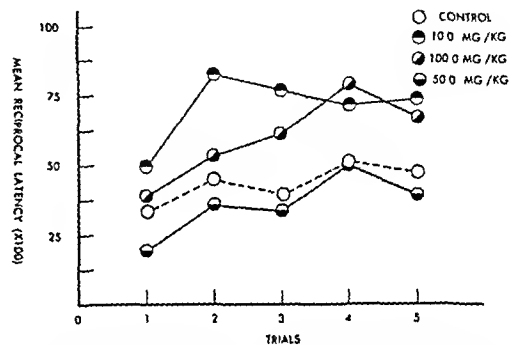


Fig. 9.—The effect of 2-ethyl-2-(3-methyl-butyl)malondiamide on avoidance-escape.

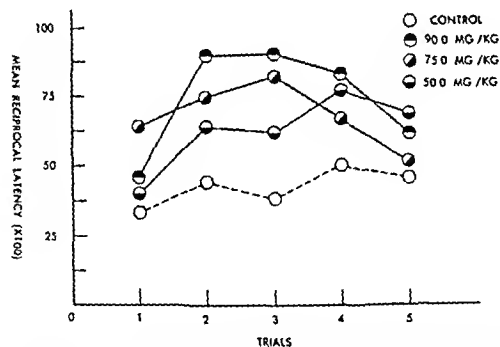


Fig. 7.—The effect of ethyl-2,4-toluene diacarbamate on avoidance-escape.

not justify a conclusion as to whether this drug interfered with the acquisition of conditioning, or whether it prevented the animal from responding. Diarrhea, and ptosis of the eyelids, in addition to sedation, were obvious in all animals receiving this drug by the third trial (in this case five days after the first trial). The highest dose of reserpine was the first to manifest these results. The activity of reserpine phosphate was found to be cumulative.

Chlorpromazine hydrochloride was found to have a significant effect on conditioning in a four mg./Kg. dose. The curve for this drug indicates that at this dose chlorpromazine appears to interfere with the

avoidance response to a greater extent than with the escape component. However, the response at this dose level is not significantly different from that for reserpine at the highest dose of the latter.

According to the *t* tests, meprobamate does not interfere significantly with conditioning at any dose level. An examination of the response curves indicates an increase in response latency for the highest dose, especially on trial days two and four. This might be consistent with the observation of pronounced flaccid paralysis in all the rats studied at this dose.

The effects of hydroxyzine hydrochloride on avoidance-escape conditioning as reflected by the *t* tests indicate a decrease in response latency at a dose of 25 mg./Kg., whereas no significant effect occurred at a dose of 35 mg./Kg. It is proposed that the highest dose used was beginning to interfere with its own activity at this point, since doses higher than 35 mg./Kg. could not be investigated because such doses caused pronounced convulsive seizures.

It is possible that amphetamine sulfate followed the same pattern of activity since similar results were obtained with this drug, i. e., the median dose facilitating the response and the stimulatory effects of the highest dose interfering with this facilitation.

On the other hand, amobarbital significantly decreased response latency at the low and median dose levels when compared to the controls. This would be consistent with the observation that barbiturates

frequently cause some stimulation prior to depression. This is further borne out by the fact that the animals receiving the highest dose of this drug showed signs of sedation and that depression on the fifth trial day was markedly present when compared to the control group.

Ethyl-2,4-toluene dicarbamate presents an anomaly of effects which are not explainable on the basis of present experimental evidence. In preliminary tests, this compound demonstrated strongly sedative properties. This does not agree with the facilitation of the conditioning response as shown by the *t* tests. Because of the apparently contradictory results achieved with this compound, a more exhaustive pharmacological study of its properties is being undertaken.

The effect of 2-ethyl-2-(3-methyl)-butylmalondiamide on avoidance-escape conditioning, as indicated by *t* tests and inspection of the curves in Fig. 8, shows, that at the lowest dose a significant decrease in latency of response resulted. It was also found that at this dose no significant difference between 2-ethyl-2-(3-methyl)-butyl malonic acid diethylester and 2-ethyl-2-(3-methyl)-butylmalondiamide was apparent. Also, no significant difference between

2-ethyl-2-(3-methyl)-butylmalondiamide and amobarbital was evident at the three doses used. Significant differences were obtained between meprobamate and 2-ethyl-2-(3-methyl)-butylmalondiamide at the high and low dose levels. However, other tests (to be published) indicated that the effects of the diamide were not unlike certain effects produced by meprobamate and amobarbital.

In general, 2-ethyl-2-(3-methyl)-butylmalonic acid diethylester showed effects similar to those produced by the corresponding diamide. In view of the fact that higher doses of the diethylester demonstrated less pronounced effects when compared to the diamide, it is felt that the former is less effective.

## REFERENCES

- (1) Kety, S. S., *Ann. N. Y. Acad. Sci.*, **66**, 3(1957).
- (2) Garattini, S., and Ghetti, V., "Psychotropic Drugs," Elsevier Publishing Co., New York, N. Y., 1957.
- (3) Cook, L., and Weidley, E., *Ann. N. Y. Acad. Sci.*, **66**, 740(1957).
- (4) Summa, A. F., and Janke, P. J., *THIS JOURNAL*, **46**, 363(1957).
- (5) Sakoda, J., Statistics Department, U. Conn., Personal Communication.
- (6) Edwards, A. L., "Experimental Design in Psychological Research," Rinehart and Co., New York, N. Y., 1954, pp. 150-151.

# The Antidotal Effectiveness of Sodium Cobaltinitrite in Antagonizing Cyanide Poisoning in Albino Mice\*

By MARVIN M. GOLDENBERG† and DAVID E. MANN, Jr.

The prophylactic intraperitoneal administration of sodium nitrite (80 mg./Kg.) thirty minutes prior to the subcutaneous injection of one LD<sub>50</sub> of sodium cyanide protected 80 per cent of the mice. Under similar circumstances, sodium cobaltinitrite (60 mg./Kg.) afforded optimal protection to 97 per cent. Both sodium nitrite and sodium cobaltinitrite, administered thirty seconds after the injection of 1 LD<sub>50</sub> of sodium cyanide, required lower doses and were more effective than when given prophylactically. The most effective therapeutic dose of sodium nitrite was 50 mg./Kg. which produced 90 per cent survival, while sodium cobaltinitrite was most effective at a dose of 30 mg./Kg., which produced a 96 per cent survival. Sodium cobaltinitrite-sodium thiosulfate combinations, when antidoting 4 LD<sub>50</sub>'s of sodium cyanide, achieved percentage results that were similar to those attained with sodium nitrite-sodium thiosulfate combinations in antidoting 3 LD<sub>50</sub>'s of the poison.

**D**ESPITE A DECLINE in the incidence of accidental and suicidal cyanide deaths during the past twenty-five years (1), the search for more effective cyanide antidotes continues. Hydrogen peroxide (2), potassium permanganate (3), glutathione and cystine (4), cobalt nitrate (5), methylene blue (6), *p*-aminopropiophenone (7),

and vitamin B<sub>12</sub> (8) are valid antagonists experimentally, but these agents failed to protect against as many median lethal doses as either sodium nitrite or sodium thiosulfate, while the combination of sodium nitrite and sodium thiosulfate, as demonstrated by Chen and Rose (9) in dogs, proved capable of antagonizing twenty median lethal doses of sodium cyanide, and accordingly was modified for clinical use.

The inception of cobalt salts as cyanide antagonists occurred in 1894 (10) when Antal protected a person against hydrocyanic acid poisoning with the oral and subcutaneous administration of eo-

\* Received August 21, 1959, from Temple University, School of Pharmacy, Philadelphia 40, Pa.

† Present address: Hahnemann Medical College, Philadelphia, Pa.

Abstracted from a thesis presented to the Graduate School of Temple University, School of Pharmacy, by Marvin M. Goldenberg in partial fulfillment of the requirements for the degree of Master of Science.

Presented to the Scientific Section, A. P. H. A., Cincinnati, meeting, August 1959.

balt nitrate. The subsequent decline in the use of cobalt salts was attributed to the introduction of other antidotes and a report by Martin and O'Brien in 1901 (11) that these agents offered no advantage over ferrous salts which, incidentally, were less toxic. In 1935, a Russian investigator (12) prophylactically protected mice against lethal doses of sodium cyanide with cobalt nitrate and cobalt sulfate. Since nitrites and cobalt salts have been demonstrated to be methemoglobin formers and sustainers, respectively, it was presumed that sodium cobaltinitrite might be useful in cyanide poisoning.

The primary purpose of this investigation was to compare sodium cobaltinitrite with sodium nitrite in terms of antidotal effectiveness when each agent was administered alone, prophylactically and therapeutically, and in conjunction with sodium thiosulfate.

## EXPERIMENTAL

Adult albino mice (Huntingdon Farms HTF strain), of mixed sex, weighing between 19.0 and 34.5 Gm. were employed in this study. The following concentrations of each agent were prepared in distilled water: sodium cyanide, 0.1 to 0.4%; sodium nitrite, 0.3 to 1.0%; sodium cobaltinitrite, 0.1 to 1.0%; and sodium thiosulfate, 25%. Solutions of sodium cyanide were administered subcutaneously, while the antidotes were injected intraperitoneally. Cessation of respiration was the criterion of death. Observations were made over a twenty-four-hour period.

**Determination of the  $LD_{50}$  of Sodium Cyanide.**—One hundred and forty-three mice were divided into five groups containing 12, 15, 6, 10, and 100 animals. Doses of sodium cyanide, ranging from 14 to 18 mg./Kg. were administered to each group in increasing increments of 1 mg./Kg. to determine a dose that would cause 100% mortality. Since only 100 animals were tested, it would be statistically incorrect to use the term minimal lethal dose<sub>100</sub> ( $MLD_{100}$ ).

**Establishment of the Minimal Prophylactic Dose of Sodium Cobaltinitrite Against 1  $LD_{50}$  of Sodium Cyanide.**—The evaluation of the effectiveness of sodium cobaltinitrite as a potential cyanide antagonist entailed the establishment of an antidotal dose which possessed a wide margin of safety. Furthermore, the optimal time interval between the administration of the antidote and the poison had to be ascertained, for methemoglobinemia produced by nitrite ion and maintained by cobalt ion (13) may attain a maximal concentration within several minutes and then drop sharply causing death from cytotoxic anoxia. Three hundred mice, half males and half females, were divided into three equal groups. Freshly prepared solutions of sodium cobaltinitrite, in concentrations of 0.1 and 1.0%, were administered to each group in successively increasing doses of 40, 50, and 60 mg./Kg., followed in thirty minutes by the administration of 1  $LD_{50}$  of sodium cyanide.

**Establishment of the Minimal Prophylactic Dose of Sodium Nitrite Against 1  $LD_{50}$  of Sodium Cyanide.**—The experimental plan in this determination

was identical to that of the previous procedure except that freshly prepared solutions of 0.5% sodium nitrite were administered to three groups of 100 mice each in doses of 60, 70, and 80 mg./Kg.

**Relative Therapeutic Effectiveness of Sodium Cobaltinitrite and Sodium Nitrite Against 1  $LD_{50}$  of Sodium Cyanide.**—The purpose of this procedure was to determine the optimal therapeutic dose for each antidote against 1  $LD_{50}$  of sodium cyanide when the poison was administered initially, followed in thirty seconds by either agent. Varying concentrations of sodium cobaltinitrite and sodium nitrite were prepared as follows according to the dose: 0.3% when the dose was 30 mg./Kg.; 0.4% for 40 mg./Kg.; and 0.5% when the dose was either 50, 60, or 90 mg./Kg. Sodium cyanide was administered as a 0.1% solution. Groups of fifty mice, of equally divided sex, were employed.

**Relative Therapeutic Effectiveness of Sodium Nitrite-Sodium Thiosulfate and Sodium Cobaltinitrite-Sodium Thiosulfate Against Increasing  $LD_{50}$ 's of Sodium Cyanide.**—Chen and Rose (9) established that sodium nitrite could antidote four median lethal doses of sodium cyanide in dogs. When sodium thiosulfate was administered in conjunction with sodium nitrite, marked potentiation occurred which increased the number of  $LD_{50}$ 's of cyanide to twenty. This study was undertaken to observe the effect of antidotal combinations against increasing  $LD_{50}$ 's of sodium cyanide. Concentrations of sodium cyanide, from 0.1 to 0.4%, were prepared and administered when the  $LD_{50}$ 's were increased from one to four. Sodium cobaltinitrite and sodium nitrite, as 0.5% solutions, were administered in doses of 60 mg./Kg. A 25% solution of sodium thiosulfate, 0.2 ml., was administered to each animal as a fixed dose. Each group consisted of fifty mice of equally divided sex. The sequence of injections was as follows: sodium cyanide was injected subcutaneously, sodium cobaltinitrite or sodium nitrite was given intraperitoneally immediately following the cyanide, sodium thiosulfate was then administered at once intraperitoneally in another portion of the abdomen.

## RESULTS

**$LD_{50}$  of Sodium Cyanide.**—The administration of sodium cyanide to four groups of mice in doses of 14, 15, 16, and 17 mg./Kg. produced survivals of 58% at the lower dosage level and 30% at the higher level. No survivals occurred at 18 mg./Kg. which was accordingly designated the  $LD_{50}$  of sodium cyanide because of the limited number of animals used.

**Minimal Prophylactic Dose of Sodium Cobaltinitrite Against 1  $LD_{50}$  of Sodium Cyanide.**—Thirty minutes was chosen as the optimal time interval between the injection of sodium cobaltinitrite and sodium cyanide, for this period appreciably increased per cent survival as well as prolonged survival time. The administration of sodium cobaltinitrite thirty minutes prior to the cyanide at a dose of 40 mg./Kg. resulted in a 47% survival with an average survival time of 50 minutes. At 50 mg./Kg., sodium cobaltinitrite produced an 85% survival with an average survival time of 56 minutes. The minimal prophylactic dose of the antidote was established at 60 mg./Kg., which produced a 97% survival with an average survival time of 58.3 minutes.

**Minimal Prophylactic Dose of Sodium Nitrite Against 1 LD<sub>50</sub> of Sodium Cyanide.**—Sodium nitrite, administered prophylactically thirty minutes prior to sodium cyanide at a dose of 60 mg /Kg produced a 13% survival with an average survival time of 49.9 minutes. At 70 mg./Kg sodium nitrite increased the per cent survival to 49 and the average survival time to 53.3 minutes. The minimal prophylactic dose, established at 80 mg /Kg., caused an 85% survival with an average survival time of 45.3 minutes.

**Therapeutic Effect of Sodium Cobaltinitrite and Sodium Nitrite Against 1 LD<sub>50</sub> of Sodium Cyanide.**—The administration of 1 LD<sub>50</sub> of sodium cyanide followed in thirty seconds by either sodium cobaltinitrite or sodium nitrite at various dosage levels produced the following results: at 60 mg /Kg, sodium cobaltinitrite protected 100% of the mice, while sodium nitrite protected 70%. At 50 mg /Kg, sodium cobaltinitrite again afforded 100% protection, while sodium nitrite increased the survival percentage to 90. At 40 and 30 mg /Kg, sodium cobaltinitrite produced survivals of 98 and 96%, while sodium nitrite decreased the survival percentage to 74 and 42, respectively.

**Sodium Nitrite-Sodium Thiosulfate and Sodium Cobaltinitrite-Sodium Thiosulfate Against Increasing LD<sub>50</sub>'s of Sodium Cyanide.**—When each antidote was administered with a fixed dose of sodium thiosulfate immediately after 1 LD<sub>50</sub> of sodium cyanide, sodium cobaltinitrite protected 100% of the mice, while sodium nitrite protected 92%. Against 2 LD<sub>50</sub>'s, sodium cobaltinitrite again showed complete protection, while only 68% of the animals were protected by sodium nitrite. Against 3 LD<sub>50</sub>'s, 80% of the mice survived with sodium cobaltinitrite protection, while only 14% were protected by sodium nitrite. Finally, against 4 LD<sub>50</sub>'s, no animals survived with sodium nitrite, while 18% were protected by sodium cobaltinitrite.

## DISCUSSION

Sodium cobaltinitrite, when administered alone prophylactically and therapeutically, and therapeutically with a fixed dose of sodium thiosulfate, proved to be a more effective antidote for cyanide poisoning in mice than sodium nitrite under similar conditions. Although sodium nitrite, when given thirty seconds after 1 LD<sub>50</sub> (18 mg /Kg) of sodium cyanide was more effective at a lower dosage level than that required for prophylactic usage, sodium cobaltinitrite produced more survivals at all therapeutic dosage levels. Thus, one may assume

that the antidotal ineptness of sodium nitrite is due to the production of methemoglobinemia of short duration, while sodium cobaltinitrite apparently has a more sustained action in perpetuating this state. This assumption is based upon the work of Shen, *et al* (13), who elucidated the probable mechanism of action of cobalt on the reversible hemoglobin-methemoglobin system. They concluded that cobalt inhibited the enzymatic reduction system concerned with the conversion of methemoglobin to hemoglobin and that the increased formation of methemoglobin was not due to the acceleration of the oxidative process beyond the capacity of the normal reduction mechanism. When they administered cobalt salts to experimental animals, increased levels of methemoglobin concurrent with a decrease in the oxygen-carrying capacity of the peripheral blood failed to appear. Therefore, one may conclude that a probable mechanism of detoxification for cobaltinitrite is the production and maintenance of methemoglobin due to the presence of the two different chemical ions.

In order for antidotes to work effectively, they should not only be capable of overcoming the toxic effects of the poison but also be relatively free of untoward effects when administered. Therefore, acute toxicity studies with sodium cobaltinitrite were observed upon a limited number of mice. It was found that the antidote produced an approximate LD<sub>50</sub> of 140 mg /Kg. Ostreiko and Charausoff (5) reported that the LD<sub>50</sub> of cobalt nitrate in rabbits was 60 mg /Kg, while Murdock and Klotz (14) found the LD<sub>50</sub> of cobalt chloride in rats to be approximately 8 mg /Kg.

## REFERENCES

- (1) Mortality Statistics, Bureau of Census, U S Dept of Commerce, 1926(1949)
- (2) Krohl, P, *Arch d pharm Inst zu Dorpal*, 7, 153(1892)
- (3) Kossa, W, *Ungar med Arch*, 2, 12(1893)
- (4) Voegtlin, C, *J Pharmacol Exptl Therap*, 27, 467(1926)
- (5) Ostreiko, O, Charausoff, H A, *Fiziol. Zhur S S S R*, 21, 643(1936)
- (6) Brooks, M M, *Am J Physiol*, 102, 145(1932)
- (7) Rose, C L, Welles, J S, Fink, R D, and Chen, K K, *J Pharmacol Exptl Therap*, 89, 109(1947)
- (8) Mushett, W, *et al*, *Proc Soc Exptl Biol Med*, 112, 234(1952)
- (9) Chen, K K, and Rose, C L, *J Am Med. Assoc.*, 149, 113(1952)
- (10) Antal, J, *Ungar, med Arch*, 3, 117(1894).
- (11) Martin, C and O'Brien, M, *Intercolon M J of Austral*, 6, 245(1901)
- (12) Rozhkov, V M, *Fiziol Zhur. S S S R*, 19, 582(1935).
- (13) Shen, C J, *et al*, *J Clin Invest*, 33, 1560(1954)
- (14) Murdock, H R, and Klotz, L J, *This Journal*, 48, 143(1959)

# A Comprehensive Pharmaceutical Stability Testing Laboratory III\*

## A Light Stability Cabinet for Evaluating the Photosensitivity of Pharmaceuticals

By LEON LACHMAN, CHARLES J. SWARTZ, and JACK COOPER

The design and utilization of a light stability cabinet for testing pharmaceuticals has been described. The lighting system developed for this cabinet is intended to simulate ordinary room illumination found in the pharmacy, home, doctor's office, or in other storage areas for pharmaceuticals, but under accelerated light intensity. The use of this equipment eliminates the influence of certain wavelengths in the ultraviolet spectrum of artificial sunlight testing equipment presently in use. Several representative pieces of artificial sunlight testing equipment used in the textile and dye industries are discussed. The photosensitivity of two tablet preparations were studied utilizing the specially designed light stability cabinet. Samples were stored in an open dish and in amber bottles. In one case the tablets contained a sulfonamide which darkened, while the other tablets contained a benzothiadiazine derivative plus D&C Orange No. 3 which showed fading of the color with storage. It was demonstrated that the amber glass appears to inhibit the deleterious effects of light on the tablets.

THE USE of more complex active constituents and certified colorants in pharmaceutical dosage forms in recent years has focused increased attention on the influence of light on the stability of these products. As a result, manufacturers of pharmaceuticals have attempted to utilize various exaggerated light testing techniques in an effort to predict the degree of photosensitivity for new formulations. However, although it is known that tests of this nature have become more prevalent, few reports exist in the literature concerning their use. This may be attributed to the fact that the methods employed were not completely satisfactory, were highly specific, and did not simulate the spectral energy distribution of ordinary room illumination.

Because of the apparent lack of satisfactory equipment for light study evaluations of pharmaceuticals, an investigation was undertaken to develop a suitable apparatus. As a result, a cabinet was designed to permit the study of dosage forms under illumination exhibiting a radiant energy distribution similar to that of ordinary room lighting, but under exaggerated light intensity. This paper reports on the photosensitivity of two tablet formulations, one containing a sulfonamide and the other a benzothiadiazine derivative plus D&C Orange No. 3. The samples were stored in the light stability cabinet so that the intensity of light falling on the products was approximately 550 foot candles. The protective

influence of amber glass containers on the tablets stored in the light cabinet was ascertained.

### PREVIOUS STUDIES

Reports in the pharmaceutical literature concerning the influence of light on the stability of dosage forms are rare. Experiments performed by Arny, *et al.* (1, 2), disclosed the effects of sunlight and diffused light on various pharmaceutical ingredients when stored in several types of glass containers. Of all the commercially available glasses studied, they found that amber glass afforded the greatest protection. The authors studied the deterioration of 50 medicaments and summarized the causes as follows: (a) light, (b) simple volatilization, and (c) chemical changes produced by factors other than light. Garrett and Carper (3) reported on color stability of a liquid multisulfate preparation as affected by thermal degradation. The influence of several pharmaceutical materials on the fading of FD&C Blue No. 2 in solution was investigated by Kuramoto, *et al.* (4). As in the previous reference (3) however, no attempt was made to isolate the contribution of the light intensity factor to the overall reaction.

Although there is a conspicuous lack of information concerning the light stability of pharmaceuticals, there appears to be an abundance of information available on the influence of light on textiles, dyes, and pigments (5-14). The necessity of having a standard light fastness test is well recognized in these fields.

The most readily available source of radiant energy is sunlight, but it is hardly necessary to point out the unsuitability of sunlight or daylight as a source of light in testing color stability. The radiation received from the sun is subject to wide variations both in total intensity and in distribution of energy. Because of this, exposure tests to sunlight result in marked differences in relative effect for samples exposed to this light source. It has been re-

\* Received June 11, 1959, from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.  
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

ported by Appel and Smith (10) that by limiting the exposure of samples to between 9 a. m. and 3 p. m. on clear days, the low intensity radiation and high atmospheric humidity occurring at intervals during continuous exposure is avoided. This, they indicate, yields data that have been shown to be generally reproducible at different times of the year and in different parts of the United States. Despite the above effort at standardization of sunlight exposure, the findings of other investigators appear to be at variance with the findings of Appel and Smith since no compensation was made for the influence of clouds, mist, or time of year.

Attempts to measure the energy of the sun's rays (15) and to make exposures for a definite number of energy units were inadequate in that the following factors were not taken into consideration: (a) variation of sun intensity from season to season and area to area, (b) daily cycles of change in temperature, (c) daily cycles of change in humidity, (d) variations in spectral character of radiation during time of exposure, (e) the influences of gases such as ozone, oxides of nitrogen, and sulfur, and (f) the presence of other atmospheric impurities. In an endeavor to concentrate the sun's rays and thereby decrease the time required for fading exposure, lenses were utilized, though unsuccessfully because of the excessive heat developed (16). In order to control, diminish, or eliminate the effect of these variables, various artificial light sources have been developed with a spectral energy distribution intended to simulate that of direct sunlight. Sources such as carbon arcs (17, 18), mercury vapor lamps (19), fadometers (6, 8, 11), daylight (10), and tungsten filament lamps (9, 12) have been used to determine the light stability of colored textiles, dyes, and chemicals susceptible to photochemical reaction.

The most commonly used reproducible high intensity point light source is the carbon arc lamp. This lamp is made in three types: flame arcs, low-intensity arcs, and high-intensity arcs. Flame arcs may be open to the air or enclosed in a glass globe. They are arranged so that a large proportion of their radiation is derived from the flame and only a comparatively small amount emitted from the tips of the electrodes. In all forms of carbon arc, the carbon electrodes burn away by oxidation and volatilization. In the enclosed arc, only a limited supply of oxygen reaches the carbons, with an ensuing burning rate which is very low. As a result, the enclosed carbon arc is the form most commonly employed in fading tests. This source resembles sunlight only to the extent that the proportion of radiant power emitted in the region 300–400  $m\mu$  is approximately the same as summer sunlight. The high intensity carbon arc is the nearest to noon sunlight in its spectral characteristics but this type of arc cannot be left to burn unattended for periods longer than a few hours. Since attempts are made to extrapolate one hundred to two hundred hours of this exposure data to several years, operation irregularities can cause large errors in extrapolated results. Further, as the high intensity arc lamp burns, the flame rotates slowly around the electrodes and, at any one time, the intensity of illumination on one side of the lamp may be three times as great as that on the opposite side.

Another artificial source frequently employed

would be mercury vapor discharge lamps, and a number of types of electric discharge lamps have been used for photochemical work. In each of these units the discharge passes through mercury vapor and the spectrum of mercury, which differs according to the operating conditions of the lamp, is emitted. In addition to supplying high intensities of irradiation, the spectrum is relatively rich in radiation throughout the blue and ultraviolet region. The advantages of this equipment are compactness, cleanliness, uniform intensity, and lower heat output for equal illumination. But the disproportionate amounts of ultraviolet light present in this source prevents the accumulation of useful information relevant to daylight fading.

A third principle light source would be the familiar tungsten filament lamp. It consists of a coil of tungsten wire mounted in a glass bulb containing an inert gas. The wire is heated to incandescence by the passage of current and the spectrum is the usual continuous type characteristic of an incandescent body. The distribution of energy of a typical high-wattage filament lamp usually commences slightly below 400  $m\mu$  and increases at a rapid rate from there. The candle power of the lamp falls slowly throughout its life, due to the volatilization of the tungsten and increased resistance of the filament.

Cabinets for housing the light source, whatever its nature, are available in a multiplicity of designs. Numerous patents (20–24) have been issued for various installations dependent upon the desired end use. The basic design consists of a housing to contain the sample at a fixed distance from a light source. A typical apparatus generally comprises a hollow cylindrical casing enclosing a source of light and having about its periphery a series of sample holders. The Fade-O-meter, a widely employed standardized unit, is based on this principle. In some instances, provisions are made for humidity and/or temperature control, while in other cases only the character of the radiation is regulated. The degree of control varies with the intended end use of the data obtained, but in almost all situations an attempt is made to extrapolate the information derived to some extended time interval.

It is readily evident from the previous sections of this report, that the artificial light sources described were intended to simulate sun illumination under controlled environmental conditions. Hitherto, consideration has not been given to the possibility of utilizing a high intensity light source which would simulate ordinary room illumination. This type of light source should more closely approximate the spectral energy distribution falling on products kept in usual storage areas. Very seldom are products exposed to direct sunlight and when they are, it is for a relatively short period of time.

## EXPERIMENTAL

**Description of Cabinet for Accelerated Light Testing.**—The magnitude of a photochemical reaction is dependent upon the amount of light energy absorbed by the material under study. The energy  $E$  absorbed by each reacting mole of material can be expressed by the following equation:

$$E = Nhc/\lambda$$

where  $N$  is the Avogadro number,  $c$  the velocity of light,  $h$  Planck's constant, and  $\lambda$  the wavelength of the absorbed radiation. Inserting the value  $N$  as  $6.023 \times 10^{23}$ ,  $h$  as  $6.624 \times 10^{-27}$  and  $c$  as  $2.9977 \times 10^{10}$ , the energy is obtained in ergs; dividing by  $4.184 \times 10^{10}$  gives the value in Keal.

$$E = 2.859 \times 10^5 / \lambda \text{ Keal. per mole}$$

It can be seen from the equation that the energy absorbed per mole becomes greater as the wavelength of the light is shortened. Therefore, the radiation in the ultraviolet and violet portions of the spectrum should be more chemically active than those of the longer wavelength (visible light). In general, photochemical activity drops off with increasing wavelength.

Since most pharmaceutical preparations are usually stored in retail pharmacies, storage areas of a warehouse, homes, hospitals, and doctor's offices, the amount of light in the ultraviolet spectrum of sunlight that penetrates through the glass store front or window panes is substantially decreased because of absorption by the glass. Husa (25) indicated that ordinary glass absorbed the shorter ultraviolet light as well as the longer infrared found in sunlight. This would suggest that the decomposition caused by sunlight passing through glass would be due to visible light and/or the ultraviolet and infrared which are nearest in wavelength to visible light.

In a report by Esselen and Barnby (26) light intensities outside and inside retail stores were measured and compared. These readings were taken in a number of midwestern stores with unobstructed southern exposure at noon on clear days in the middle of June. The values obtained from such exposure should represent the most severe sunlight exposure conditions obtainable. Foot candle readings of intensity were made both inside and outside the store front with the meter pointing directly south. The data obtained from this study are reproduced in Table I. It is readily evident from the data that the outdoor readings were over 400 times the average reading taken on typical store shelves exposed to light of normal intensity.

TABLE I.—LIGHT INTENSITY OF TYPICAL RETAIL STORE LOCATIONS<sup>a</sup>

Meter Location	Average Light Intensity, Foot candles	Outside Reading/ Inside Reading
Outside store windows	6,500	
Shelves, front of store	15-30	216 to 433
Shelves, middle of store	5-15	1,300 to 433
Shelves, shaded parts of store	1-5	6,500 to 1,300

<sup>a</sup> See reference 26.

In view of the preceding discussion, it would seem that in order to measure the effect of light on the sensitivity of pharmaceuticals accurately, a lighting system should be developed which simulates ordinary room illumination but under accelerated light intensity. Through the use of such equipment the influence of certain wavelengths in the ultraviolet spectrum of artificial sunlight sources which are generally not present in storage areas for pharmaceuticals would be eliminated.

It is desirable to utilize high intensity light for studies of a photochemical nature, since the chemical change produced by light is generally small and develops at a slow rate. For example, it has been calculated that radiation of one candle power falling on 1 sq. cm. corresponds to  $2 \times 10^{14}$  quanta per second, so that this number of molecules should react per second. The number of moles reacting per second per candle power is therefore  $3.3 \times 10^{-10}$  per sq. cm. of substance exposed to radiation (27).

In consideration of the above, a light stability cabinet was fabricated to reproduce ordinary room illumination, but under substantially intensified light. A brief preliminary discussion of this cabinet was presented in an earlier paper (28). Figure 1 gives a comprehensive description of this cabinet with the lighting system employed and its specifications.

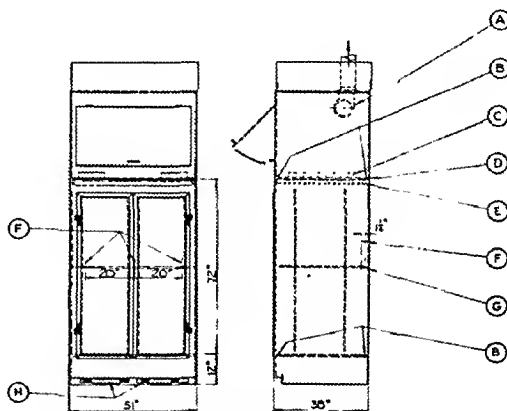


Fig. 1.—Comprehensive description of the lighting cabinet used for exaggerated light stability testing. A, Exhaust blower; B, slit front and rear (air supply); C, ballast, (9) No. 89G604-G.E.-150 watts, each ballast is wired with (2) No. 48T12 fluorescent tubes; D, Alzac aluminum sheet reflector above lamp tubes; E, rapid start lamps, (18), No. 48T12 CWRs, 1 1/2 inches in diameter x 48 inches long slant line cool white, G.E., rated for 3,250 lumens each 60 watt tubes. Ground tubes minimum of 1/2 to 1 inch from mounting surface. (9) G.E. No. 505 x 91 socket, (9) G.E. No. 505 x 92 socket; F, (3) thermocouples on these center lines; G, Alzac aluminum shelf (adjustable); H, grill for air supply.

The fluorescent tubes used are rated for 3250 lumens each and are of the slant line cool white, rapid starter type. They are fabricated to give off the least amount of heat per foot candles of light intensity produced. Any heat that emanates from the fluorescent tubes is removed by means of a blower located above the ballasts containing the tubes. A curve representing the spectral distribution of energy in typical sunlight as it reaches the earth's surface and in the emission of a "standard cool white" fluorescent lamp is presented in Fig. 2. The curves have been adjusted so that each represents the same amount of light. It can be seen that the ratio of ultraviolet radiation to visible light is greatest for sunlight and substantially less for the "standard cool white" lamp. Nevertheless, it should be emphasized that light intensity within buildings is manyfold less than in direct sunlight.

Alzac aluminum has been used for the sample tray



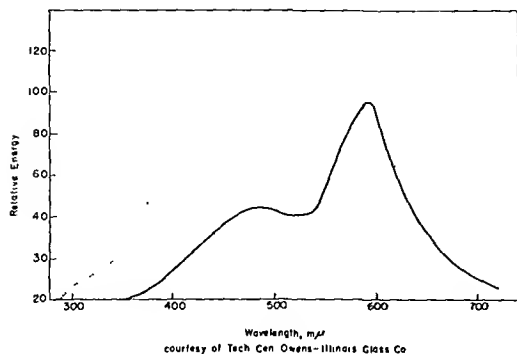


Fig. 2.—Spectral energy distribution of sunlight and standard cool white fluorescent lamp . . . Sun, — standard cool white fluorescent.

as well as behind the fluorescent tubes for reflectance purposes.

The ballasts provide for proper starting voltage and also control the operating voltage and current in the tubes.

The shelf in the cabinet is adjustable so that it can be moved to any desired height. This adjustment makes it possible to obtain variable light intensities, since the light intensity is apparently inversely proportional to the square of the distance.

Through the use of this cabinet it is possible to obtain a reproducible light intensity over a definite spectral range at any time during the year under controlled conditions of temperature and humidity. This should permit the determination of the stability of pharmaceuticals with regard to light in a relatively short period of time.

**Description of Light Stability Cabinet for Ordinary Illumination.**—This cabinet has been fabricated to simulate room illumination intensity and spectral energy distribution. Samples are stored in this cabinet at the same time they are exposed to accelerated light intensity. The dimensions of this cabinet are 37 inches high by 18 inches deep by 30 inches long. The light sources in this cabinet are two 8-watt and two 15-watt fluorescent tubes which can be used independently or in combinations to attain the desired light intensity. The interior of the cabinet is painted a flat white for reflectance purposes. The cabinet permits the exposure of samples to room illumination under specified conditions and acts as a control for exaggerated intensity studies.

The light meter employed for intensity measurements is a Gossen Tri Lux foot candle meter model C which has a range of measurement from 0–12,000 foot candles. It is an incident meter measuring the light falling on an object. The accuracy of this meter is  $\pm 5\%$  of the end scale value.

**Procedure for Testing Products.**—Samples of two different tablet formulations, one containing a sulfonamide and the other a benzothiadiazine plus D&C Orange No. 3 as colorant were exposed to 550 foot candles of light intensity. These tablets were held in an uncovered container and in an amber bottle. Reflectance measurements were performed on the tablets at designated time intervals utilizing a model DU Beckman spectrophotometer with a reflectance attachment. Special dies were prepared to hold the tablets in the reflectance attachment. Reflectance

measurements for the sulfonamide tablet were performed at 450  $m\mu$  and for the tablet containing the benzothiadiazine at 500  $m\mu$ .

**Results and Discussion.**—The light intensity falling on the materials being tested was maintained at a constant level by regular measurement with the Gossen foot candle meter followed by necessary adjustments in the shelf distance from the source of light. In an earlier report (28) it was shown that it is possible to calculate the foot candles of light intensity falling on a test material with the aid of a suitable equation.

A summary of the results obtained for the sulfonamide tablets is presented in Table II. It is evident from this data that the tablets darken with storage since the reflectance values decrease substantially. Visual observation of these tablets indicate that they have taken on a yellow-tan color. The darkening of the tablets can probably be ascribed to oxidation of the sulfonamide which is potentiated by the light. However, for samples stored in amber bottles no significant change in reflectance values from the original 52.6% was noted. The amber glass appears to protect the sulfonamide tablets from the harmful effects of the light in the cabinet. The data in this table were plotted on semilog paper and the curves are shown in Fig. 3. By plotting the log of the per cent reflectance *vs.* time, straight line curves are obtained. For the samples exposed directly to the intense light a curve results which has two slopes, the first being steeper than the second.

The effect of light on the tablets containing the benzothiadiazine derivative colored with D&C

TABLE II.—INFLUENCE OF ACCELERATED LIGHT STORAGE ON THE PHOTSENSITIVITY OF A SULFONAMIDE TABLET FORMULATION

Time, Wks	Reflectance at 450 $m\mu$ , %	
	Open Dish	Amber Bottle
0	52.6	52.6
2	41.3	52.3
4	35.6	53.6
6	31.7	53.8
8	28.9	52.9
10	25.4	...
12	23.8	51.5

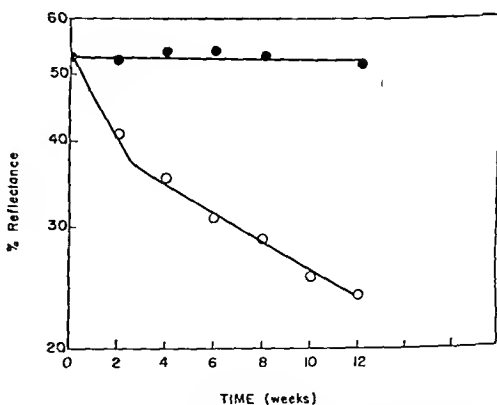


Fig. 3.—A plot showing the influence of light on the color stability of a sulfonamide tablet. ● Tablets stored in amber bottle, ○ tablets stored in open dish.

TABLE III. INFLUENCE OF ACCELERATED LIGHT STORAGE ON THE PHOTSENSITIVITY OF A TABLET FORMULATION CONTAINING A BENZOTHIADIAZINE DERIVATIVE AND D&C ORANGE No. 3

Time, Wks.	Absorbance at 500 $m\mu$	
	Open Dish	Amber Bottle
0	0.302	0.302
2	0.234	0.302
4	0.212	0.315
6	0.203	0.304
8	0.188	0.312
10	0.198	0.312
12	0.190	0.309

Orange No. 3 is shown in Table III. It can be readily seen from the data in this table that the color of the tablets exposed directly to the intense light fades significantly as indicated by an increase in reflectance values. Again, the samples stored in amber bottles show essentially no change with storage. By plotting the logarithm of the absorbance vs. time, straight line curves are obtained as shown in Fig. 4. The plot for the sample exposed directly to the intense source of light shows three different slopes. Since the plots are straight lines, it would seem that the fading follows an apparent first-order reaction. Between seven and eight weeks, the curve levels off and shows no further fading. Because of these interesting data, a more comprehensive study was initiated concerning the influence of ordinary room illumination and exaggerated lighting on the rate of fading of several commonly used certified colorants. The results obtained from this study will be presented in a separate report.

The protective characteristics of amber glass becomes readily evident from the transmittance curves for flint and amber glass shown in Fig. 5. While flint glass transmits significantly from 300  $m\mu$ , the amber glass does not begin to transmit to any appreciable extent until 470  $m\mu$ . This would indicate that the energy emitted by cool white fluorescent lamps in the light cabinet beyond 470  $m\mu$  does not exert any appreciable effect on the samples tested. Since it is believed that reactions of particular chemicals are effected by a narrow band of wavelengths near the absorption maximum of the chemical (11), it may be anticipated that the influence of the light source would vary according to the effective range of wavelengths present.

A more complete study on the influence of various colored glass and thickness on the light stability of a number of pharmaceutical components is under way and will be reported on in the near future.

According to the data presented in this report as well as that obtained from additional studies performed in our laboratories, it would appear that although a relationship exists between the extent of a reaction and light intensity of ordinary and exaggerated illumination, this relationship is not independent of the material undergoing testing. In other words, the exaggeration produced by the high light intensity for one chemical could be tenfold while for another chemical only threefold. However, the extent of the reaction for each chemical is generally assumed to be dependent on the product of time and light intensity (11). These differences in degree of exaggeration that are produced for different chemicals can be ascribed to several factors: (a) the wave-

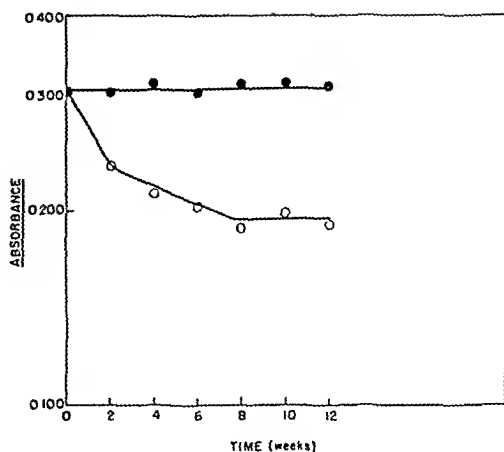


Fig. 4.—The effect of light on the fading of a tablet colored with D&C Orange No. 3. ● Tablets stored in amber bottle, ○ tablets stored in open dish.

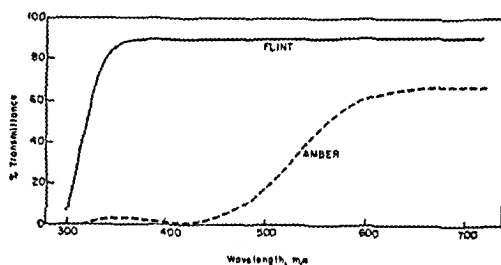


Fig. 5.—Transmission curves for typical flint and amber glass.

lengths of the spectrum which are absorbed, (b) the amount of electromagnetic radiation of a suitable wavelength required to cause the molecules of a chemical to reach its excited state, and (c) the amount of radiation absorbed. Therefore, it seems most appropriate to determine the extent of exaggeration of a reaction produced by the high light intensity employed in the light stability cabinet for each product studied and not attempt to apply data from previous tests on similar products until sufficient studies have been performed to indicate the feasibility of this approach.

## SUMMARY AND CONCLUSIONS

The design and application of a light stability cabinet for testing the photosensitivity of pharmaceutical products has been described. The lighting system employed for this cabinet is believed to simulate ordinary room illumination but under accelerated light intensity.

Data are presented for the effect of light on the stability of a sulfonamide and a benzothiadiazine tablet formulation. The colorant used for the benzothiadiazine tablet was D&C Orange No. 3.

The influence of amber glass on the light stability of the two tablet formulations was deter-

mined. It was shown that although the lighting in the cabinet exerted significant deleterious effects on the color of the tablets stored in an open dish, these changes were virtually prevented when the tablets were stored in amber containers.

### REFERENCES

- (1) Arny, H. V., Taub, A., and Blythe, R. H., *THIS JOURNAL*, 23, 672(1934)
- (2) Arny, H. V., Taub, A., and Steinberg, A., *ibid.*, 20, 1014(1931)
- (3) Garrett, E. R., and Carper, R. F., *ibid.*, 44, 515(1955)
- (4) Kuramoto, R., Lachman, L., and Cooper, J., *ibid.*, 47, 175(1958)
- (5) Lips, H. A., *Paper Trade J.*, 120, 108(1954)
- (6) Nordhammer, G., and Gralen, N., *J. Soc. Dyers Colourists*, 65, 741(1949)
- (7) Cady, W. H., *Am. Dyestuff Repr.*, 20, 130(1931)
- (8) Cady, W. H., and Appel, W. D., *ibid.*, 18, 407(1929)
- (9) Luchesh, M., and Taylor, A. H., *ibid.*, 14, 613(1925)
- (10) Appel, W. D., and Smith, W. C., *ibid.*, 17, 410(1928)
- (11) Morton, T. H., *J. Soc. Dyers Colourists*, 65, 597(1949)
- (12) Cooper, B. S., and Hawkins, F. S., *ibid.*, 65, 586(1949)
- (13) Vickerstaff, T., and Tough, D., *ibid.*, 65, 606(1949)
- (14) Hadfield, I. H., *J. Textile Inst.*, 18, 527(1927)
- (15) Appel, W. D., *Am. Dyestuff Repr.*, 16, 715(1927)
- (16) Gordon, C., *Textile Colorist*, 43, 29(1921)
- (17) Olinger, C. G., *Am. Dyestuff Repr.*, 31, 28(1942)
- (18) Lead, W. L., *J. Soc. Dyers Colourists*, 65, 723(1949)
- (19) Blaisdell, B. E., *ibid.*, 65, 618(1949)
- (20) Buttolph, L. J., U. S. pat. 1,818,687, August 11, 1931
- (21) British pat. 435,097, September 13, 1935
- (22) British pat. 390,731, April 13, 1933
- (23) British pat. 325,112, February 13, 1930
- (24) Stott, L., U. S. pat. 1,661,332, March 27, 1928
- (25) Husa, W. J., *Glass Container*, 7, 9(1928)
- (26) Esselen, W. B., and Barnby, H. A., *Modern Packaging*, 1939, September
- (27) Glasstone, S., "Textbook of Physical Chemistry," D. Van Nostrand Co., Inc., New York, N. Y., 1954, p. 1159
- (28) Lachman, L., and Cooper, J., *THIS JOURNAL*, 48, 226(1959)

## A Preliminary Pharmacologic Investigation of the Roots of *Bixa orellana*\*

By NORRIS W. DUNHAM and KENTON R. ALLARD

Pharmacologic studies were made of a partially purified water extract of the roots of *Bixa orellana*. Tests showed that the extract produces a marked depressant effect on voluntary activity without impairing involuntary activity. It also possesses antisecretory, antispasmodic, and hypotensive activity.

**B**IXA ORELLANA is a plant indigenous to Central and South American countries. This plant is also called Achiote and has been used as a source of annatto, a yellow-red pigment, which is obtained from the seeds. The bush will grow to a height of 20 feet and is cultivated to yield the pigment and to serve as a wind-breaker at the edges of coffee and cocoa plantations. So far as is known, there has been no previous attempt to extract chemical or pharmaceutical products from the roots.

### EXPERIMENTAL

**Extraction Procedure.**—The whole root was gathered, ground, and extracted in a large Soxhlet apparatus with various solvents. After defatting with petroleum ether and ethyl ether, the sample was extracted with 95% ethyl alcohol for twelve hours and

the solvent evaporated spontaneously. A 4% yield of a brown powder with a melting point of 60–70° was obtained. The extraction process was repeated for twelve hours employing distilled water. The water was then evaporated with the aid of a flash evaporator and finally in a vacuum desiccator under partial vacuum. A 7% yield of a brown powder with a melting point of 180–190° was obtained.

**Effects on Spontaneous Motor Activity.**—The Aetophotometer (Metro Industries) was employed in this study. Six groups of 6 male, albino mice of the Rockland-Swiss strain were injected intraperitoneally at each of 4-dose levels. Thirty minutes after injection of an aqueous solution of the water extract, the group was placed in the photocell activity cage and their movements were recorded for the first five minutes only. The testing was repeated on different mice injected intraperitoneally with an aqueous solution of chlorpromazine HCl. Control experiments were made at random times during the tests with intraperitoneal injections of 0.2 ml of distilled water.

The percentage inhibitions were calculated and the ED<sub>50</sub> (effective dose<sub>50</sub>) were interpolated from a dose-response curve constructed on semilogarithmic paper relating log dose and percentage inhibition. The results are shown in Table I.

TABLE I—CENTRAL NERVOUS SYSTEM ACTIVITY (MICE, I. P.)

Drug	Dose, mg/Kg	Photo cell	Roller
Bixa-water extract	21	ED <sub>50</sub>	On
Chlorpromazine HCl	2	ED <sub>50</sub>	Off

\* Received August 21, 1959, from the Division of Pharmacy, Ferris Institute, Big Rapids, Mich. Supported, in part, by a grant from the Upjohn Co. Roots supplied by Harold Hartgerink, Holland, Mich. Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

**Effects on Rolling Roller Activity.**—The rolling roller apparatus originally reported by Dunham and Miya (1) as a method for detecting neurotoxicity has more recently been reported by Kinnard and Carr (2) as a method of distinguishing between various types of tranquilizing effects. At a roller speed of 5 r p m, a normal animal can maintain its equilibrium for an indefinite period. Certain central nervous system depressants will result in the animal being unable to remain on the roller for the two-minute test period.

This testing was done immediately after removing the mice from the Aetophotometer. The results are also presented in Table I. It is evident from the table that the dose of chlorpromazine required to inhibit the spontaneous motor activity by 50% also incapacitates the animal to the extent that it cannot remain on the roller. In contrast to this result, the ED<sub>50</sub> dose of the water extract of *Biva* does not exhibit the undesirable central nervous system depression. According to this method of testing, toxicity of the water extract is not observed until the lethal dose range is reached.

**Effects on Unstimulated Gastric Secretions.**—A method similar to that reported by Risley, *et al* (3), was followed employing six albino rats per dose. All drugs were injected intraduodenally in a dose of 400 mg /Kg at the time of pyloric ligations. All drugs were administered suspended in 2% tragacanth and the controls received comparable amounts of the suspending agent only. Five hours after the operation, the animals were sacrificed and the gastric juice was collected. The effects on volume, free acidity, and total acidity are shown in Table II.

It is evident that the whole root and the fractions will inhibit the volume of gastric secretion. However, only the whole root and the alcohol fraction have an effect on acidity.

TABLE II—ANTISECRETORY EFFECTS  
(RATS, 400 MG /KG, I D)

Drug	Mean Vol Secreted ml	Mean Free Acidity Meq /L	Mean Total Acidity Meq /L
Controls	6.4	41	58
Whole root	3.1	15	32
Water extract	3.4	35	59
Alcohol extract	1.6	10	35

**Effects on Intestinal Motility, In Vitro.**—Segments of guinea pig ileum were suspended in a 50 ml aerated bath of Tyrode's solution at 38°. The spontaneous contractions were recorded kymographically. The addition of 50 mg of the water extract resulted in a decrease in tonus and a cessation of contractions. At the end of approximately one minute, the rate and amplitude of contractions returned to normal but the tonus was still decreased. A similar pattern was noted when 100 mg doses were added to the bath.

**Effects on Blood Pressure.**—Rats were anesthetized by the intraperitoneal administration of pen-

tobarbital sodium (50 mg /Kg). The carotid artery was cannulated and connected to a mercury manometer with polyethylene tubing filled with a solution of heparin in normal saline. The solutions of the water extract were injected into the femoral vein. Doses of 10 mg /Kg and 25 mg /Kg resulted in transient drops in blood pressure. However, doses of 50 mg /Kg and 100 mg /Kg resulted in the pressure falling to 40% of normal and remaining there for an average of thirty minutes. The pressure then gradually increased and was normal again after sixty to ninety minutes.

**Miscellaneous CNS Testing.**—Intraperitoneal doses of 20 mg /Kg of the water extract did not potentiate pentobarbital induced sleep time in mice.

Swinyard's techniques (4) using maximal electroshock and pentylenetetrazol were employed in testing the water extract for anticonvulsant properties. Intraperitoneal doses of 100 mg /Kg in mice did not alter the convulsions produced which indicates absence of anticonvulsant activity.

**Acute Toxicity.**—Albino male mice, 18–22 Gm of the Rockland Swiss strain were employed for this determination. All animals were fasted but received water *ad libitum* for twelve hours prior to the test. The water extract was injected intraperitoneally and the animals were kept under observation for five days since some delayed deaths were noted two and three days after injection. At the end of this period, deaths were counted and the LD<sub>50</sub> was determined by Behrens' method (5). The LD<sub>50</sub> was calculated to be 700 mg /Kg.

SUMMARY AND CONCLUSIONS

These studies indicate the presence of pharmacologically active principles in the roots of *Biva orellana*. The water extract has a pronounced depressant effect on voluntary activity (spontaneous motor activity) in the mouse without impairing the involuntary activity (rolling roller performance).

The intraperitoneal ED<sub>50</sub> on depressing spontaneous motor activity is 21 mg /Kg as compared to the intraperitoneal LD<sub>50</sub> of 700 mg /Kg.

This extract inhibits the volume of unstimulated gastric secretions without influencing the acidity. It also possesses antispasmodic and hypotensive properties.

Anticonvulsant and potentiation of barbiturate induced sleep activities are absent.

REFERENCES

(1) Dunham, N. W., and Miya, T. S., *THIS JOURNAL*, 46, 208(1957).  
(2) Kinnard, W. J. and Carr, C. J., *J. Pharmacol. Exptl. Therap.* 121, 354(1957).  
(3) Risley, E. H., *et al.* *Am. J. Physiol.* 150, 754(1947).  
(4) Swinyard, E. A., *THIS JOURNAL* 38, 201(1949).  
(5) Behrens, B., *Arch. exptl. Pathol. Pharmacol.*, 140, 237 (1929).

# An Investigation of Some Pharmaceutical Applications of Certain Fatty Acid Esters of Sucrose\*

By HOWARD HOPKINS† and LA VERNE D. SMALL

The usefulness of sucrose monomyristate and sucrose dipalmitate in formulating oral preparations has been studied. The monoester, being quite hydrophilic, is useful as an emulsifier. It is not, however, sufficiently hydrophilic to solubilize volatile oils. The diester, though water-insoluble, is dispersible in hot syrups yielding gel-like products suitable for use as vehicles. Although both the monoester and the diester are claimed to be nontoxic orally, the monoester exhibits toxic effects parenterally. The intraperitoneal  $LD_{50}$  in the rat was determined. An improvement in the method of synthesis of the monoester is described. Formulations for preparation of the gel-like vehicles are suggested.

RECENT DEVELOPMENTS in sucrochemistry (1-21) have made available a new group of compounds known as the sucrose esters of the higher fatty acids. More specifically, these substances are mono- and diacyl derivatives of sucrose where the acyl group contains from 12-18 carbon atoms in the aliphatic chain (22-24). These new substances are nonionic, surface-active materials. The claim that these compounds were tasteless and nontoxic (1, 3, 6-11, 13, 14, 24-28), in contrast with hitherto available surfactants (26, 29-31), was suggestive that these new compounds might be especially useful in the food and pharmaceutical industries as emulsifiers, solubilizers, and dispersing agents. In pharmaceutical practice, these esters might (a) aid in overcoming some of the problems in the solubilization of flavoring materials and other chemicals occurring in pharmaceutical products, (b) serve as emulsifying agents in preparing emulsions for oral and parenteral use, and (c) provide the basis for the development of a vehicle or carrier for therapeutic agents.

This study was initiated to investigate the range of usefulness of certain of these compounds in the formulation of pharmaceutical products intended for oral administration. Two of these esters, sucrose monomyristate and sucrose dipalmitate, have been studied. The latter material was procured from a commercial source<sup>1</sup> but the

former was synthesized in this laboratory using a modification of the procedure proposed by Osipow, *et al.* (15). Sucrose monomyristate, a water-soluble, nonionic surfactant, was studied for its ability to act as an emulsifying and solubilizing agent. The growth behavior of a number of microbial agents in solutions of sucrose monomyristate was examined for the purpose of determining whether this sugar ester would be readily attacked by some commonly encountered types of contaminants. The intraperitoneal  $LD_{50}$  for the rat was determined for sucrose monomyristate. Sucrose dipalmitate, a water-insoluble compound, was found to be useful in formulating favorable, gel-like compositions suitable as vehicles for a variety of medications.

## EXPERIMENTAL

**Synthesis of Sucrose Monoester.**—The Osipow procedure (15) for synthesizing the sucrose monoesters involves reacting, under reduced pressure, the methyl ester of the appropriate fatty acid with sucrose in a solvent of dimethylformamide in the presence of an alkaline catalyst. At completion of the reaction, the solvent is removed by distillation under reduced pressure, the residue is dissolved in water, and this solution is then extracted with 1-butanol. Distillation of the 1-butanol from this extract leaves a residue which, on repeated reprecipitation from acetone, supposedly yields the desired sucrose monoester. In this laboratory, this procedure always gave final products which were bitter. By making the aqueous solution alkaline (pH 11) prior to extraction with 1-butanol and by extracting the 1-butanol solution with acidulated water (pH 1), a tasteless sucrose monomyristate was obtained in a 17% yield. It was found that the soap, present in the residue obtained after distillation of the 1-butanol, could be removed from the reaction mixture residue if a sufficiently large (15 to 1) amount of acetone were used in the reprecipitation of sucrose monomyristate. At a 5-10% concentration of

\* Received August 21, 1959, from the College of Pharmacy, University of Nebraska, Lincoln.

Abstracted from a thesis submitted to the Graduate College of the University of Nebraska in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Fellow of the American Foundation for Pharmaceutical Education, College of Pharmacy, University of Nebraska, Lincoln. Present address: College of Pharmacy, University of Kentucky, Lexington.

The authors wish to thank Dr. C. E. Georgi for his counsel and aid in the microbiological tests and to acknowledge with gratitude the helpful suggestions and assistance of Dr. R. D. Gibson in the toxicity determination.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

<sup>1</sup> Millmaster Chemical Corp., 295 Madison Ave., New York 17, N. Y.

sucrose monomyristate in hot acetone, the soap is not solubilized and may be filtered off

Efforts to synthesize sucrose monopalmitate from sucrose and sucrose dipalmitate, in the manner suggested for an alternate procedure (15), were wholly unsuccessful.

**Sucrose Monomyristate as a Solubilizer.**—One-milliliter quantities of a number of volatile oils, such as orange oil, methyl salicylate, and clove oil, were added to 100-ml volumes of various concentrations (1-8%) of sucrose monomyristate, agitating and mixing thoroughly, followed by measurement of the amounts of oil solubilized. An aqueous 8% sucrose monomyristate solution was incapable of completely solubilizing any of the volatile oils which were used. On the other hand, Tween 80<sup>2</sup> in 8% concentration was capable of solubilizing these volatile oils in water. The HLB (hydrophilic-lipophilic balance) value of sucrose monomyristate, calculated according to the method of Griffin (32), is 11.7 while that of Tween 80 is 15.0

Applewhite, *et al* (33), reported that Tween 80 would solubilize phenobarbital in water. Sucrose monomyristate was tested for this capacity. The test was made by adding phenobarbital in 0.1-Gm amounts to 5% aqueous solutions of Tween 80 and sucrose monomyristate to the point of saturation of each solution. For six hours after the addition of the last portions of phenobarbital to these solutions they were maintained at 25° and stirred mechanically. The 100 ml of 5% Tween 80 solution dissolved more than 0.5 Gm but less than 0.6 Gm of phenobarbital. The 100 ml of 5% sucrose monomyristate dissolved more than 0.3 Gm but less than 0.4 Gm of phenobarbital. Phenobarbital is soluble normally to the extent of 0.1 Gm in 100 ml of water.

**Emulsification Tests.**—The "required HLB value" for mineral oil has been reported to be 10.5 (34), and 12 (35). Sucrose monomyristate, with HLB value of 11.7, was tested for its capacity to emulsify liquid petrolatum. Excellent emulsions were obtained using as little as 0.4% of sucrose monomyristate. A typical formulation was as follows:

Sucrose monomyristate	2.5 Gm
Water	80.0 ml
Methyl salicylate	0.2 ml
Liquid petrolatum (heavy)	100.0 ml
Sucrose	20.0 Gm
Water	200.0 ml
Liquid petrolatum (heavy)	200.0 ml

A primary emulsion was prepared by dissolving the sucrose monomyristate in 80 ml of water, dispersing therein the methyl salicylate by mechanical stirring, adding 100 ml of liquid petrolatum while stirring vigorously, and finally dissolving the sugar in the mixture which was then forced through a hand homogenizer<sup>3</sup>. The remaining quantities of water and liquid petrolatum were added alternately in small amounts to the primary emulsion while stirring vigorously. The resulting mixture was then forced through the hand homogenizer, obtaining thereby an excellent, very palatable, and quite fluid emulsion.

The use of sucrose monomyristate as the sole

emulsifying agent for vegetable oils, such as sesame, peanut, and cottonseed oil, produced less stable emulsions than those obtained when some sucrose dipalmitate was included in the formulation. Sucrose dipalmitate, being more lipophilic than the monoester, has an HLB value of 7.5. It is, therefore, a poor o/w emulsifier when used singly. It was found, for example, that an emulsion of cottonseed oil, capable of being autoclaved without destruction of the emulsion, could be prepared using a mixture of sucrose monomyristate and Sucrodet D-600<sup>4</sup> (sucrose dipalmitate) as the emulsifier. An emulsion for parenteral use would need to be sterilized. The following formulation yielded an emulsion, capable of being autoclaved, which might be useful in parenteral alimentation:

Sucrose monomyristate	0.85 Gm.
Dextrose	9.20 Gm.
Water	170.00 ml.
Sucrodet D-600	0.15 Gm.
Cottonseed oil	30.00 ml.

The sucrose monomyristate and dextrose were dissolved in the water, heating and stirring until solution was complete. The Sucrodet D-600 was similarly dissolved in the cottonseed oil. The hot (90°) oil solution was added slowly to the hot (90°) aqueous phase, stirring the mixture vigorously during the addition of the oil and for several minutes afterward. When slightly cooled, the pH of the mixture was adjusted to 7 with sodium hydroxide solution and then forced through the hand homogenizer, packaged, and autoclaved at 15-lb pressure for twenty minutes.

**Gelled Vehicle Preparation.**—The sucrose diesters are not water soluble. It was found that an excellent dispersion of Sucrodet D-600 could be obtained in a syrup base. This dispersion was obtained by incorporating a hot (70°), alcoholic solution of the diester into hot (70°) syrup U S P, stirring until the mixture cooled to 40° or less. When the diester content was about 5%, the product obtained had an ointment-like consistency. It was gel-like since it had no noticeable tendency to flow. For purpose of identification in this section such "gelled" products are called syrup-diester dispersions.

By varying the concentration of the diester, the consistency of the syrup-diester dispersion may be controlled. The composition and consistency of these syrup-diester dispersions suggested their possible usefulness as pharmaceutical vehicles. These dispersions may be colored and flavored to produce very pleasing vehicles. Such vehicles prepared with syrup U S P, when exposed to the air, had a marked tendency to form a crystalline layer on the surface. The replacement of a portion of the syrup U S P with Sorbo<sup>5</sup> (sorbitol solution, N F) eliminated this problem. The basic, unflavored formulation which was developed was as follows:

Sucrodet D-600	10 Gm.
Alcohol	10 ml.
Sorbo	50 ml.
Syrup U S P	150 ml.

The Sucrodet D-600 and the alcohol were mixed

<sup>4</sup> Trademark of Millmaster Chemical Corp., New York, N. Y.

<sup>5</sup> Trademark of Atlas Powder Co., Wilmington, Del.

<sup>1</sup> Trademark of Atlas Powder Co., Wilmington, Del.

<sup>2</sup> Arthur H. Thomas Co., Philadelphia, Pa., No. 4308 S.

and heated to near boiling and added with vigorous stirring to a hot (70°) mixture of the Sorbo and syrup U. S. P., mixing until cool (below 40°).

Volatile oil flavors may be incorporated into these gelled vehicles by adding them to the alcohol-Sucrodet D-600 solution. If a flavored syrup is used, it may replace an equivalent volume of syrup U. S. P. For fruit-flavored products, the inclusion of some tartaric or citric acid is desirable. The gelled vehicle is adaptable to many flavor variations.

A variety of flavored gelled vehicles have been prepared. Some typical formulations appear in Table I.

TABLE I.—FLAVORED GELLED VEHICLE FORMULATIONS

Ingredients	Formulation				
	1	2	3	4	5
Sucrodet D-600, Gm.	10	10	10	10	10
Alcohol, ml.	10	10	10	10	10
Cherry syrup, ml.	50				
Raspberry syrup, ml.		50			
Chocolate syrup, ml.			50		
Tartaric acid, Gm.		1			
Citric acid, Gm.					1
Lemon oil					q s.
Vanillin				q s.	
Color	q s.	q s.	q s.	q s.	q s.
Sorbo, ml.	50	50	50	50	50
Syrup U. S. P., ml.	100	100	100	150	150

**Microbiological Tests.**—The fact that the sucrose esters contain a carbohydrate material suggested that preparations containing these esters might be prone to microbiological attack. The growth response of a number of frequently encountered microbiological contaminants was determined in media containing 0.5% and 1% sucrose monomyristate. Both of these media were tested singly and with an added nitrogen source appropriate for the organism being tested. No attempt was made to assure sterility of these media, since autoclaving of the ester solutions might have hydrolyzed a portion of the material. Effort was made to minimize contamination by sterilizing all equipment used, as well as the distilled water, and by carrying out all operations with the usual aseptic precautions observed in good bacteriological technique. The chemicals which were added to the sterile water in preparing the media were not sterilized. All media had pH values between 5 and 6. The inoculum in each instance, except for *Proteus vulgaris*, consisted of a suspension made by adding 8 ml. of sterile water to a thirty-six-hour agar-slant culture of the appropriate organism, and then mixing the surface growth into the water, as uniformly as possible. The inoculum for *Proteus vulgaris* was a thirty-six-hour nutrient broth culture of the organism. Besides *Proteus vulgaris*, the other organisms tested for growth response were *Saccharomyces cerevisiae*, *Aspergillus niger*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas fluorescens*. The volume of inoculum added to each 10-ml. sample of medium was 0.5 ml. The inoculated samples were incubated for six days at a temperature appropriate for the organism being tested. The results of these tests are summarized in Table II. The four yeast-inoculated cultures shown in Table II as having exhibited no growth at the end of six days

TABLE II.—SIX-DAY MICROBIAL GROWTH RESPONSE IN SUCROSE MONOMYRISTATE SOLUTIONS

Organism	Nitrogen Source	Sucrose Monomyristate Solution			
		0.5% Test	1.0% Control	0.5% Test	1.0% Control
<i>Saccharomyces cerevisiae</i> incubated at 27°	None HN <sub>4</sub> Cl 0.1%, K <sub>2</sub> HPO <sub>4</sub> 0.1%	— <sup>a</sup>	—	—	—
<i>Proteus vulgaris</i> incubated at 37°	None Peptone 0.5%	— <sup>b</sup>	—	+	—
<i>Aspergillus niger</i> incubated at 27°	None HN <sub>4</sub> Cl 0.1%, K <sub>2</sub> HPO <sub>4</sub> 0.1%	—	—	—	—
<i>Bacillus subtilis</i> incubated at 37°	None Peptone 0.5%	+	—	+	—
<i>Escherichia coli</i> incubated at 37°	None (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 0.1%	—	—	—	—
<i>Pseudomonas fluorescens</i> incubated at 27°	None Peptone 0.5%	+	—	+	—

<sup>a</sup> No growth (—). <sup>b</sup> Growth (+)

were separately subcultured in malt extract broth. These subcultures were then incubated at 27° for six days. At the end of this incubation period, there was substantial growth present in each subculture, indicating that the yeast organisms had survived in the presence of low surface tension during the initial six-day incubation period.

**Toxicity Tests.**—Conflicting reports (1, 26, 27), without supporting experimental data, exist relative to the parenteral toxicity of the sucrose monoesters. For sucrose monomyristate, the intraperitoneal acute toxicity determination was made using the rat as the test animal. The animals used were five-week-old, Holtzman strain, first generation rats raised at the University of Nebraska. They had been numbered and sex separated at three weeks of age. Food was withheld for twenty-four hours prior to use; water was allowed *ad libitum*. A constant dose-volume of 10 ml./Kg. was injected intraperitoneally. Nine dosage levels were used, ranging from 100 mg. to 1,662 mg./Kg. All solutions injected were made isotonic by addition of sodium chloride as required. Five males and five females were injected at each dosage level, except No. 9 at which level five adult, unfasted, female Holtzman strain rats were used. After injection of the dose, measured to the nearest 0.01 ml., each rat was individually caged in a room maintained at 26-27°, deprived of water, and observed for twenty-four hours. The results from the injections are found in Table III.

The animals in the first four groups, or dosage levels, shown in Table III, exhibited no apparent evidence of toxicity. At dosage level No. 5, the animals became somewhat sedated; at No. 6 they became lethargic. The animals in groups 7, 8, and 9, although lethargic, evidenced considerable discomfort. Shortly prior to death, pronounced cyanosis of the feet, ears, and oral cavity developed.

TABLE III.—SUMMARY OF MORTALITY OF RATS TO INTRAPERITONEAL INJECTION OF SUCROSE MONOMYRISTATE

Group No.	Dosage level, mg./Kg.	No. of Rats	Elapsed Time Until death, hr. Min.	Max.	Mortality, %
1	100.0	10	..	..	0
2	142.1	10	..	..	0
3	201.9	10	..	..	0
4	286.9	10	..	..	0
5	407.7	10	..	..	0
6	579.3	10	3.03	21.07	50—LD <sub>50</sub>
7	823.1	10	1.87	2.30	100
8	1117.1	9	1.56	1.93	100
9	1662.0	5	2.13	2.25	100

Death was attributed to anoxia with respiratory paralysis, as the heart continued to beat for a short time after respiration ceased. An autopsy of the expired animals revealed the presence of 2-5 ml. of pink-tinted to blood-tinted serous exudate in the abdominal cavity. The lungs were darker than normal with small hemorrhagic spots on the external surface. The blood vessels of the viscera were filled with dark, chocolate-colored blood. All other tissues and organs appeared to be essentially normal. One rat in group No. 6 succumbing twenty-one hours after injection evidenced a sloughing of the peritoneum. One rat in group No. 8 survived the entire test period, appearing to be quite normal. After twenty-four hours, the animal was sacrificed and upon autopsy appeared normal in every way except for some small hemorrhagic spots on the surface of the lungs. Injection into this rat must have been made into the lumen of the intestine. This animal was, therefore, discarded from the data.

Sucrose monomyristate was tested *in vitro* for its ability to hemolyze rat red blood corpuscles. About 2 ml. of blood were drawn by intracardial puncture from an etherized female rat randomly selected from the unused supply of rats remaining after the intraperitoneal toxicity study. One hundred units of heparin sodium injection U. S. P. were added to this blood. A sample of this heparinized blood was placed on a microscope slide and observed through a microscope as a small amount of 1% solution of sucrose monomyristate, previously made isotonic by the addition of sodium chloride, was brought in contact with the blood. Hemolysis of erythrocytes occurred promptly. When a drop or two of the 1% solution of sucrose monomyristate was added to about 1 ml. of the heparinized blood, the mixture was converted quickly into a clear, cherry-red solution, indicating hemolysis.

## DISCUSSION

In the synthesis of sucrose monoesters by the procedure followed in this study, it has been postulated (15) that the methyl ester of the fatty acid reacts quite rapidly with sucrose to form diesters. These diesters, in the presence of a large excess of sucrose, react with the sucrose to yield essentially pure monoester material, with the 6-position of the glucose moiety of sucrose being esterified. This reaction mechanism was the basis of the unsuccessful alternate synthesis. The reasons for this failure are not understood. The conversion of preformed

diester and sucrose into monoester should be, presumably, little different from the conversion of diester into monoester in the reaction sequence involving the methyl ester and sucrose.

Although the sucrose monoesters have been reported (26, 27) as being useful as solubilizers of vitamins A and D, their general usefulness as solubilizers appears to be quite limited. Sucrose monomyristate was disappointing in its solubilizing ability. It behaved, however, in accordance with its calculated HLB value.

In contradistinction to the success achieved in this laboratory with these sucrose esters as emulsifying agents, some workers have reported (36, 37) unsatisfactory results in attempts to use sucrose monoesters as emulsifying agents for lipid materials. In view of the results of the acute intraperitoneal toxicity test, the intravenous administration of emulsions containing sucrose monomyristate should be approached with caution.

The gelled vehicles appear to be very suitable for the incorporation of powdered materials. These vehicles can be suitably flavored to meet a variety of demands. This dosage form may be packaged in collapsible tubes and could prove to be a convenience to parents in administering medication to children. In addition, the problem of spilled medicines on the bed linens, etc., should be minimized with such a gelled dosage form. These gelled vehicles developed from Sucrodet D-600 are seemingly quite versatile and could have a rather wide range of applications.

The microbiological tests indicate that no unusual or extraordinary precautions against microbial contamination are necessary because of the inclusion of sucrose monomyristate in aqueous preparations or formulations. Spoilage, due to microbial growth, of such preparations was not observed. No study was made relative to the problem of preserving any of these preparations. Although surfactants depending upon a polyoxyethylene grouping for water solubility have been reported (38-43) as inactivating phenolic types of preservatives, it has been asserted (28, 44) that the sucrose esters do not inactivate phenolic preservatives. One study (45), however, appears to contradict this conclusion. The matter of selecting suitable preservatives to use with the sucrose esters remains unsettled.

The sucrose monomyristate solutions which were injected intraperitoneally into rats contained, at the lowest toxic concentration, 10 to 20 times more monoester than did the emulsions prepared for parenteral use. The intravenous administration of a surfactant as a component of an emulsion system might be less toxic than when administered singly in identical concentration, due to the fact that in the emulsion the surfactant is part of a somewhat fixed system. Thus, the surfactant in an emulsion might not be instantly available to produce toxic effects. This question was not investigated. However, the intravenous toxicity of sucrose monomyristate should be determined by an animal study.

## SUMMARY

1. An improved procedure for preparing the sucrose mono-fatty acid esters has been developed. This process eliminates bitter-tasting substances present in the final product pre-



pared by the previously recommended procedure

2. Sucrose monomyristate has been found to be a poor solubilizer for essential oils, thereby reducing its potential usefulness in pharmaceutical formulation

3. The sucrose esters used in this study are good emulsifying agents. Mixtures of the mono-ester and diester were more suitable for emulsifying vegetable oils than either of the esters when used singly. The mixtures may be more effective due to better approximation of the required HLB of the oils. Sucrose monomyristate produced excellent liquid petrolatum emulsions

4. The calculated HLB values for the sucrose esters used in this study appear to be in agreement with experimental findings of this study

5. Sucrose dipalmitate, Sucrodet D-600, in a syrup base produced an excellent semisolid, gelled vehicle. This vehicle may be flavored as desired. These flavored, gel-like vehicles are

recommended as being well-suited to pediatric use

6. The sucrose esters used in this study do not appear to favor microbial growth

7. Intraperitoneal acute toxicity was determined for sucrose monomyristate using the rat. The LD<sub>50</sub> was 579 mg./Kg. Sucrose monomyristate produced rapid hemolysis of rat blood *in vitro* at low concentrations.

8. Although the sucrose fatty acid esters may have what appears to be a rather limited range of usefulness due to their inability to solubilize certain materials effectively, nevertheless, they do appear to be well-suited for use as emulsifying agents and for the preparation of inexpensive, favorable, gel-like vehicles which are suitable for use with many therapeutic agents

9. These new, surface-active materials appear to possess sufficient pharmaceutical potential to warrant a complete toxicological study

## REFERENCES

- (1) Hass, H. B., *Chemurgic Dig.*, 14, No. 6, 8 (1955)
- (2) Anon., *Mfg. Chemist*, 24, 368 (1953)
- (3) Anon., *Chem. Week*, 75, Sept. 11, 49 (1954)
- (4) Anon., *ibid.*, 77, Oct. 8, 90 (1955)
- (5) Pacini, A. B., *Drug and Allied Inds.*, 41, Dec., 12 (1955)
- (6) Anon., *Drug & Cosmetic Ind.*, 78, 239 (1956)
- (7) Hass, H. B., *Chem. in Can.*, 8, April, 39 (1956)
- (8) Anon., *Drug and Allied Inds.*, 42, Dec., 15 (1956)
- (9) Osipow, L., *J. Soc. Cosmetic Chemists*, 7, 249 (1956)
- (10) Pacini, A. B., *Drug and Allied Inds.*, 43, May, 4 (1957)
- (11) Anon., *Dodge & Olcott News*, 10, Aug., 4 (1957)
- (12) Ancona, A., *Boll. chim. farm.*, 97, 401 (1958)
- (13) Osipow, L., Marra, D., and Snell, F. D., *Drug & Cosmetic Ind.*, 80, 312 (1957)
- (14) Hass, H. B., *Mfg. Chem.*, 29, 152 (1958)
- (15) Osipow, L., Snell, F. D., York, W. C., and Finchler, A., *Ind. Eng. Chem.*, 48, 1459 (1956)
- (16) Anon., *Drug & Cosmetic Ind.*, 79, 824 (1956)
- (17) Anon., *Ind. Eng. Chem.*, 48, Oct., 13A (1956)
- (18) Anon., *Chem. Eng. News*, 35, April 29, 108 (1957)
- (19) Anon., *ibid.*, 35, May 27, 80 (1957)
- (20) Anon., *ibid.*, 35, July 8, 78 (1957)
- (21) Anon., *ibid.*, 36, April 21, 24 (1958)
- (22) York, W. C., Finchler, A., Osipow, L., and Snell, F. D., *Intern. Congr. Pure and Appl. Chem.*, XIV Congr., Zurich, Switzerland, July 21-27, 1955, through *Ind. Eng. Chem.*, 48, 1459 (1956)
- (23) York, W. C., Finchler, A., Osipow, L., and Snell, F. D., *J. Am. Oil Chemists' Soc.*, 33, 424 (1956)
- (24) Anon., "Sucrodet's General Information Brochure," and "Sucrodet D 600 Technical Data Sheet," Millmaster Chemical Corp., 295 Madison Avenue, New York 17, N. Y., August 1957
- (25) Osipow, L., Snell, F. D., Marra, D., and York, W. C., *Ind. Eng. Chem.*, 48, 1462 (1956)
- (26) Mima, H., *Pharm. Bull. (Tokyo)*, 5, 496 (1957)
- (27) Mima, H., *Yakugaku Zasshi*, 78, 988 (1958), through *Chem. Abstr.*, 53, 1636 (1959)
- (28) Raphael, L., *Mfg. Chem.*, 29, 237 (1958)
- (29) Fitzhugh, O. G., and Nelson, A. A., *THIS JOURNAL*, 37, 29 (1948)
- (30) Lambert, G. F., Miller, J. P., and Frost, D. V., *ibid.*, 45, 685 (1956)
- (31) Raphael, L., *Mfg. Chem.*, 29, 105 (1958)
- (32) Griffin, W. C., *J. Soc. Cosmetic Chemists*, 5, 349 (1954)
- (33) Applewhite, R. W., Buckley, A. P., and Nobles, W. L., *J. Am. Pharm. Assoc., Pract. Pharm. Ed.*, 15, 164 (1954)
- (34) Anon., "Atlas Surface Active Agents," Atlas Powder Co., Wilmington, Del., 1950, p. 28
- (35) Anon., "Guide to the Use of Atlas Surfactants and Sorbitol in Pharmaceutical Products," Atlas Powder Co., Wilmington, Del., 1958, p. 22
- (36) Tober, T. W., and Autian, J., *J. Am. Pharm. Assoc., Pract. Pharm. Ed.*, 19, 422 (1958)
- (37) Hom, F. S., "The Preparation and Stabilization Studies of Ethiodol Emulsions for Intravenous Hepatolienography," Thesis, Temple University, Philadelphia, Pa., 1956 pp. 16-20
- (38) deNavarre, M. G., *Congr. mondial detergente et prods. tensio actifs, 1<sup>er</sup> Congr., Paris*, 2, 741 (1954), through *Chem. Abstr.*, 51, 14294 (1957)
- (39) deNavarre, M. G., and Bailey, H. E., *J. Soc. Cosmetic Chemists*, 7, 427 (1956), through *Chem. Abstr.*, 51, 1549 (1957)
- (40) Hall, C. S., and deNavarre, M. G., *Proc. Soc. Sect. Toilet Goods Assoc.*, 27, 29 (1957), through *Chem. Abstr.*, 51, 11659 (1957)
- (41) Popprzan, J., and deNavarre, M. G., *J. Soc. Cosmetic Chemists*, 10, 81 (1959)
- (42) Mulley, B. A., and Metcalf, A. D., *J. Pharm. and Pharmacol.*, 8, 774 (1956)
- (43) Barr, M., and Tice, L. F., *THIS JOURNAL*, 46, 445 (1957)
- (44) Moore, C. D., and Hardwick, R. B., *Mfg. Chem.*, 29, 194 (1958)
- (45) Wedderburn, D. L., *J. Soc. Cosmetic Chemists*, 9, 210 (1958)

# The Effects of Tranquilizers on Bacterial Toxemias I. Reserpine\*

By LEO GREENBERG, JAMES W. INGALLS, and ARTHUR G. ZUPKO

Purified exotoxins of *Clostridium tetani* and *Clostridium botulinum* are highly lethal to mice. Pretreatment of experimental animals with a single, large dose of reserpine prior to toxin administration resulted in a highly significant prolongation of survival time. Similar prolongation was achieved in overwhelming infection induced with *Diplococcus pneumoniae*. This effect was not demonstrated with small doses of reserpine administered in a divided series either prior to or concurrent with the infection.

IT is a widely accepted concept today that bacterial infection places a stress upon the diseased animal. In view of the potent pharmacological activity of the tranquilizers in ameliorating many of the stress factors of modern living, it seemed of interest to investigate what effects, if any, these drugs might have on disease processes of bacterial origin. Such studies have now been under way at our laboratories since 1957, and the present paper deals with the role of reserpine in some of bacterial stress situations.

Although studies of reserpine action have been largely confined to the area of emotional or psychological stress, several references to its role in physiological stress have appeared in the literature. Thus, it is known that the drug is of value in protecting animals against hemorrhagic shock (1), while in experimental *Staphylococcus aureus* infection it may exacerbate the infectious process through the depletion of 5-hydroxytryptamine (2). It has also been reported that in a dose of 5 mcg., reserpine increased the toxicity of *Neisseria gonorrhoeae* endotoxin for mice, while at a dose of 2 mcg., no significant effects were observed (3). On the other hand, a significant prolongation of survival time from *Clostridium botulinum* toxin with 10 mcg. reserpine has been noted in mice (4).

## MATERIALS AND METHODS

Male mice weighing approximately 25 Gm. were used throughout these experiments. Preliminary experiments with divided doses of reserpine and the pneumococcal studies were done with CFW and Rockefeller strain animals. All other data were derived from CF1 (Carworth Farms) animals. Mice were caged in small groups and kept in the thermostatically-controlled animal house for several days prior to use.

Reserpine solution<sup>1</sup> was diluted with distilled

water and all inoculations were made by intraperitoneal injection. Except where otherwise indicated, the drug was injected approximately forty-five minutes prior to introduction of the toxic agent.

Tetanus toxin<sup>2</sup> in 0.85 NaCl solution with 0.3 M glycine, an Lf. titer of 870/ml., and a mouse LD<sub>50</sub> of 112 million was used throughout. Purified *Clostridium botulinum* type A Toxin<sup>3</sup> diluted with two parts of glycerol and a mouse LD<sub>50</sub> of approximately  $5 \times 10^6$ /ml. was used. Toxins were stored in the refrigerator, the botulinus material at  $-20^\circ$  to maintain potency. All dilutions were made with sterile isotonic saline. Toxin injections were always in a volume of 0.1 ml. and were administered intraperitoneally.

*Diplococcus pneumoniae* strain SVI was obtained as a lyophilized culture from the Rockefeller Institute and grown in brain-heart infusion broth (Difco). Slants were maintained on brain heart infusion agar with added blood. Subculturing was done weekly and virulence maintained by monthly mouse passage. For inoculations, an eighteen-hour broth culture containing approximately  $2 \times 10^8$  organisms was used, injections being by the intraperitoneal route.

In all experiments, paired groups of animals of the same weight, age, and source were used and all control and experimental animals in any given run were inoculated within a few minutes of one another. None of the diluent substances used showed any toxicity to mice, and sterile brain heart infusion broth injections were shown to be without effect.

## RESULTS AND DISCUSSION

**Toxin Studies.**—A highly significant prolongation of life in both tetanus and botulinus toxemia can be seen from the data in Table I. It will be noted, however, that the combination of agents used is a specific one—a large dose of the tranquilizer and an overwhelmingly lethal dose of the toxin. This combination was determined after preliminary experiments with tetanus toxin involving more than 600 mice indicated that this approach was the most fruitful, yielding consistently reproducible results. Other approaches investigated included:

**Use of Highly Dilute Toxin.**—In control animals it was determined that 50% of the animals injected with various dilutions of toxin died as follows:

\* Received August 21, 1959, from the Research Institute of the Brooklyn College of Pharmacy, Long Island University, Brooklyn 16, N. Y.

Supported in part by a grant from Wallace Laboratories, New Brunswick, N. J.

Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.

<sup>1</sup> Supplied as Serpasil through the courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J.

<sup>2</sup> Supplied through the courtesy of Dr. H. A. Dettwiler, Eli Lilly & Co., Indianapolis, Ind.

<sup>3</sup> Supplied through the courtesy of Matteo Cardella, Immunology Branch, U. S. Army Biological Warfare Laboratories, Fort Detrick, Frederick, Md.

TABLE I—EFFECT OF RESERPINE ON SURVIVAL TIME FROM TETANUS AND BOTULINUS TOXINS

Treatment—		No of Animals	Mean Survival Time, min	Significance
Toxin	Reserpine mg /Kg			
0.1 ml, 1:100				
Botulinus	0	10	129.3 ± 6.1	Difference between control and both experimental groups is highly significant, P < 0.001
Botulinus	2.0	10	163.4 ± 9.8	
Botulinus	2.0	10	161.4 ± 8.6	
Tetanus	0	80	151.0 ± 7	Difference not significant, P > 0.4 Difference is significant, P < 0.02
Tetanus	0.2	10	183.7 ± 39	
Tetanus	2.0	10	275.3 ± 43	
Tetanus	0	20	108.1 ± 5.5	Differences are both highly significant, P < 0.001
Tetanus	2.0	10	226.3 ± 16	
Tetanus	3.0	10	224.2 ± 17	

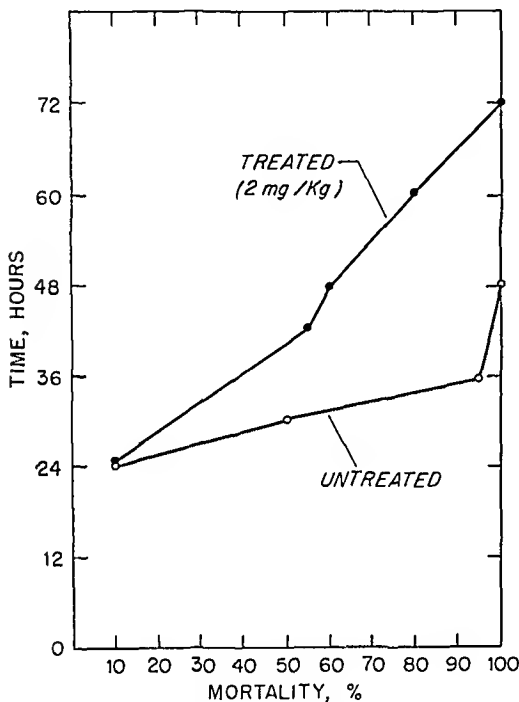


Fig. 1.—Effect of reserpine on survival time from pneumococcal bacteremia.

1:50,000/twenty-four hours; 1:100,000/thirty hours; 1:150,000/thirty-six hours; 1:200,000/forty-four hours; 1:250,000/seventy-two hours, 1:300,000/one hundred and twenty hours. A single dose of 0.1 mg./Kg. reserpine failed to show any significant departure from these survival figures.

**Pretreatment with Repeated Reserpine Injections.**—In 50 animals injected with 1:400,000 toxin, 54% were dead within twelve hours. Daily injections of 0.1 mg./Kg. reserpine for five days prior to toxin administration resulted in an apparent increased lethality of the toxin, 80% of the animals being dead within twelve hours. Likewise, when three injections of 0.1 mg./Kg. reserpine were given eight hours apart for twenty-four hours prior to toxin introduction, no significant changes in survival time were found in groups of ten animals each at toxin dilutions of 1:2,000; 1:5,000; 1:10,000; 1:20,000; 1:50,000; 1:100,000; 1:150,000; 1:200,000; 1:250,000; 1:300,000.

**Use of Repeated Post-toxin Reserpine Injections**—Groups of ten animals were given toxin dilutions of 1:200,000, 1:300,000; 1:400,000, and 1:500,000. Reserpine at 0.1 mg./Kg. was given immediately thereafter and once daily until the death of the animal. At all levels, a trend toward increased survival was noted, but results were without statistical significance.

**Pneumococcal Studies.**—As in the case of the toxin studies previously outlined, the combination of a large dose of reserpine administered approximately forty-five minutes prior to the introduction of a toxic agent of overwhelming lethality, in this case an undiluted broth culture of virulent pneumococci, resulted in a highly significant prolongation of survival time. A typical response is shown in Fig. 1.

Use of undiluted broth culture was decided upon after ten weeks of preliminary testing. It was found that the limits of survival time of mice could be more sharply defined when undiluted culture was used than when any dilution of the culture was employed. Dilution of the culture in sterile broth or saline prolonged survival time compared to undiluted culture, but did not prolong it reliably in proportion to the dilution.

In 29 control groups of ten mice, each injected with 0.1 ml. of undiluted broth culture of pneumococci, 90 to 100% of the animals died between the sixteenth and forty-eighth hour. Pretreatment with 0.2 mg./Kg. reserpine, either prior to or subsequent to infection, revealed no gross deviation in survival patterns. When the dose of reserpine was increased to 2.0 mg./Kg. or more, significant prolongations of survival time could be demonstrated repeatedly, and apparently the extent of such prolongation is directly proportional to the dose employed, being limited at its upper limits by the toxicity of the reserpine itself.

SUMMARY AND CONCLUSIONS

1. Groups of mice were injected with overwhelmingly lethal doses of three toxic agents, *Clostridium tetani* exotoxin, *Clostridium botulinum* exotoxin, and *Diplococcus pneumoniae* broth culture.
2. Pretreatment forty-five minutes prior to the introduction of the toxic agent with a single dose of reserpine, 2 mg./Kg. or higher, resulted in highly significant prolongation of survival time.
3. Neither post-toxin treatment with reserpine

nor pretreatment with small divided doses evidenced this effect in our experiments.

4. In addition to its proved usefulness in emotional and psychological stress, reserpine is apparently capable of favorably influencing the course of bacterial stress in laboratory animals.

## REFERENCES

- (1) Fine, J., Frank, E. D., Ravin, H. A., Rutenberg, S. H., and Schweinburg, F. B., *New Engl. J. Med.*, 260, 214(1959).
- (2) Mishra, B. P., and Sanyal, R. K., *J. Pharm. and Pharmacol.*, 11, 127(1959).
- (3) Tauber, H., and Garson, W., *Proc. Soc. Exptl. Biol. Med.*, 97, 886(1958).
- (4) Boroff, D. A., *Intern. Arch. Allergy Appl. Immunol.*, 15, 74(1959).

## Binding of Drugs by Plastics II\*

### Interaction of Weak Organic Acids With Plastic Syringes

By H. K. KIM and J. AUTIAN

Three plastic syringes were evaluated as to their ability to bind a number of weak organic acids. Polyethylene and polystyrene syringes did not interact with any of the seven agents included in the binding study. Benzoic acid, *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid were bound to various degrees by the nylon syringes. No interaction was noted with any of the syringes and acetylsalicylic acid, phenobarbital, or barbital. The degree of binding was influenced by concentration, temperature, and diffusion.

THE RAPID INCREASE in the use of plastics in place of glass, rubber, and metals is very evident by observing the multitude of products now composed, in whole or part, of plastic. Today, in many instances, a plastic may be formulated to convey certain outstanding properties to a product while minimizing objectionable properties. The success of these plastic materials in practically all fields has created in the minds of many a confidence in plastics which is a tribute to the scientific achievements of the plastic industry.

It is rather unfortunate that this "confidence" is now engendered by certain groups in both the medical and pharmaceutical field. Since plastics is still relatively in its infancy in medical and pharmaceutical practice, it is not too surprising to see or hear of instances where plastic materials have been utilized without proper evaluation first taking place. Usually, there is no problem when an ethical pharmaceutical firm utilizes a plastic material for a container, syringe, or tubing, since extensive tests are conducted to insure that the plastic material has no unusual effect upon the specific drug system to be used in the plastic device. Some concern, however, should be elicited by those clinicians or pharmacists who are, or will be utilizing plastic devices for various medicinal products, i. e., syringes, tubings, etc. In this respect, Bryant and Brewer (1) have suggested

that new toxicity and safety tests should be initiated when disposable pharmaceutical materials are to be employed.

Recently reports have appeared which point out some of the difficulties encountered in the use of plastic devices with certain medicinal preparations.

Autian and Brewer (2) noted in their study of plastic-hubbed needles that a certain plastic formulation released a toxic constituent to saline solution. They further found that a solvent (diethyl carbonate) in a particular parenteral product dissolved the plastic hubs.

In the evaluation of three types of plastic syringes (polyethylene, polystyrene, and nylon) as to their physical compatibility with parenteral products, a number of incompatibilities were seen with the polystyrene but not with the other two plastic materials (3).

Certain plastic tubings to be used as medical grade tubing were found to leach acidic constituents to drug solutions (4). It was rather interesting to note that in certain solutions heavy leaching occurred; whereas in others only minor or no leaching occurred.

Still another problem has been encountered with the use of plastics in syringes. In this case, Marcus, *et al.* (5), reported that nylon syringes bound to various degrees, a number of bacteriostatic agents. They found that parahydroxybenzoic acid, methylparaben, propylparaben, sorbic acid, phenol, and 4-chloro-3-methylphenol were bound by the nylon.

\* Received September 10, 1959, from the College of Pharmacy, University of Michigan, Ann Arbor.

Presented to Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

This research project was conducted under a grant from the Becton, Dickinson and Co. Rutherford, N. J.

Even though the number of incompatibilities appear to be rather limited, the brief review above should indicate that incompatibilities may occur which cannot be easily detected by the clinician or nurse. This is especially true of those cases where plastic materials bind the drug and thus prevent the prescribed dose being administered to the patient.

This paper continues the study previously reported (5) on the binding of drugs by plastics. In this particular study a number of weak organic acids was included to determine if one or more of the drugs would be bound by the three types of plastic syringes.

## EXPERIMENTAL

### Apparatus and Reagents

**Apparatus.**—Beckman DU spectrophotometer; Beckman pH meter, model G; and constant temperature ovens.

**Plastic Syringes.**—Polyethylenec, Becton, Dickinson and Co., Rutherford, N. J.; polystyrenec, Becton, Dickinson and Co.; nylon, Atlas Surgical Supplies, Ltd., Manchester, England, distributed in U. S. by Child's Surgical Supply, Inc., Pasadena, Calif.

**Reagents.**—Benzoic acid, U. S. P., *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, N. F., salicylic acid, R. G., acetylsalicylic acid, U. S. P., phenobarbital U. S. P., barbitol N. F.

### Methods of Analysis

**Benzoic Acid, *m*-Hydroxybenzoic Acid and Salicylic Acid.**—A titration procedure was employed to determine the quantity of these acids. A standard barium hydroxide solution (0.05 *N*) was used as the titrant and phenolphthalein as the indicator.

**Acetylsalicylic Acid.**—The quantitative analysis of aspirin was conducted by employing a titration procedure using a standard alcoholic potassium hydroxide solution (0.02 *N*) as the titrant and  $\alpha$ -naphtholbenzein as the indicator.

***p*-Aminobenzoic Acid.**—A spectrophotometric method was used to determine the absorbance of *p*-aminobenzoic acid at a wave length of 270  $m\mu$  and in a concentration of 1 mg./100 ml. Distilled water was employed as a blank.

**Phenobarbital and Barbitol.**—Both drugs were assayed by a spectrophotometric method at a wave length of 240  $m\mu$  in a borate buffer system (pH 9.4). Borate buffer solution was used as the blank, and the concentrations employed for both barbiturates in the cell were adjusted to a strength of 1 mg./100 ml.

### Binding as a Function of Concentration and Temperature

Two-milliliter size syringes were employed in this study with the needles removed and the tip at the junction of the canula and barrel fused by application of heat.

The binding experiments were essentially those reported in a previous paper (5). Four concentrations<sup>1</sup> for each agent were prepared using double-

distilled water as the solvent. Each solution was then added to each barrel, stoppered with a cork encased in parafilm, and stored for a period of forty-eight hours at a specified temperature (5, 30, and 50°  $\pm$  1°). Three barrels were employed for each concentration and a control sample (in a Pyrex test tube).

After the storage period, the barrels were removed and the contents pooled for each concentration (and for each drug). The pooled samples were then assayed for their respective drugs and the results calculated as the average value for one barrel.

Neither the polyethylene nor the polystyrene barrels bound, to any degree, the drugs included in this study. Four of the seven drugs were bound, to various degrees, by the nylon barrels. These were benzoic acid, *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid. Figures 1-4 relate the quantity of bound drug to the quantity unbound.

Acetylsalicylic acid and the two barbituric acids were not bound, to any extent, by the nylon barrels.

### Binding as a Function of Time

Solutions were prepared of the eight drugs and placed into each type of plastic barrel, stoppered, and stored at 30°. Sufficient barrels were employed to insure that duplicate samples of each drug could be assayed over a period of one week.

As before, benzoic acid, *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid were bound by the nylon barrels. Figure 5 contains the results for the week period (in hours) expressed as drug bound (in per cent). The nylon barrels did not

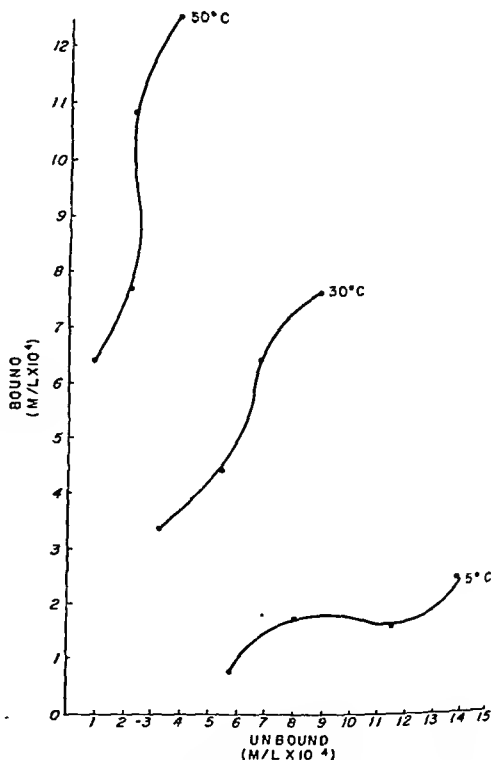


Fig. 1.—Binding of benzoic acid by nylon syringes.

<sup>1</sup> 0.001 *M* H<sub>2</sub>SO<sub>4</sub> was used to suppress hydrolysis in the aspirin solution.

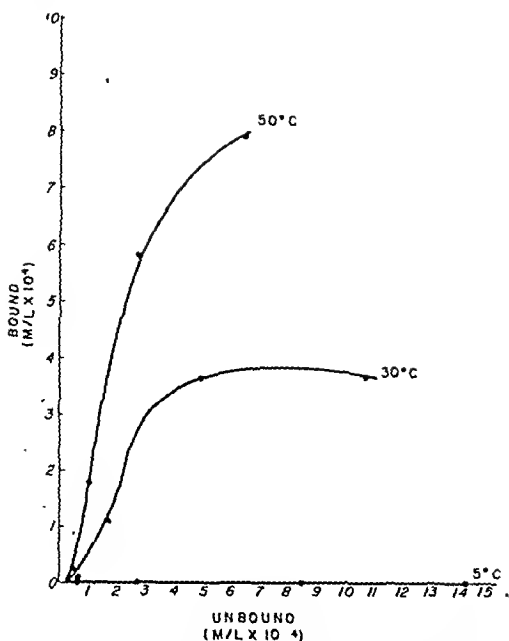


Fig. 2.—Binding of *m*-hydroxybenzoic acid by nylon syringes.

bind the other three drugs. None of the drugs were bound by either the polyethylene or polystyrene barrels.

## DISCUSSION

Even though it was assumed that no binding would occur with either the polyethylene or polystyrene barrels because of the lack of polar centers in the macromolecules, these two types of barrels were included in the study to detect if other physical or chemical reactions would occur. As reported, no such reaction was observed for any of the drugs included in the experiments within the one-week period.

Previous investigation with nylon barrels (5) confirmed earlier thoughts that nylon syringes would bind or sorb certain drugs possessing acidic hydrogens. The carbonyl groups of the polyamide structure would be expected to attract those molecules possessing proton-donating groups such as phenols and weak organic acids. The energy required for binding appears to be primarily due to dipole-dipole interaction between the reactants; however, secondary attraction forces (van der Waals) must contribute sufficient energy to achieve a stable interaction (binding). It has been pointed out that dipole-dipole interaction alone would not produce stable binding, since very polar water molecules would also be competing with the acidic molecules for the binding sites in the polyamide (6). The relatively large concentration of water molecules to the acidic molecules would favor statistically the interaction of the water molecules to the negative sites in the polyamide.

As may be seen from Figs. 1–5, binding occurred with four drugs (benzoic acid, *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid) of the seven weak organic acids. In each instance,

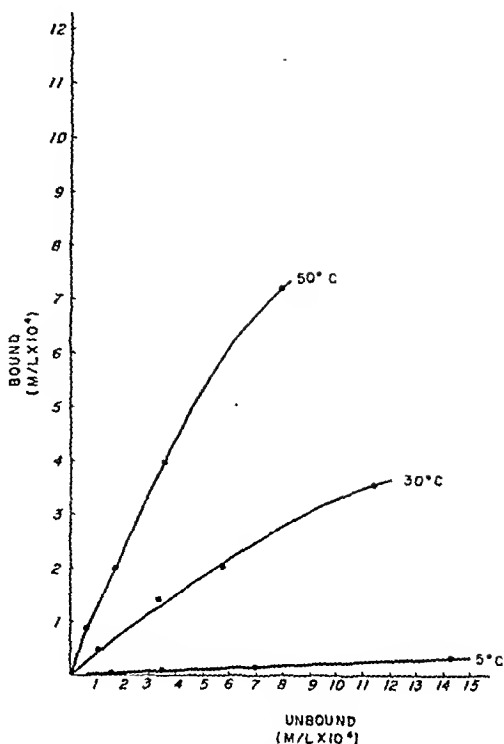


Fig. 3.—Binding of *p*-aminobenzoic acid by nylon syringes.

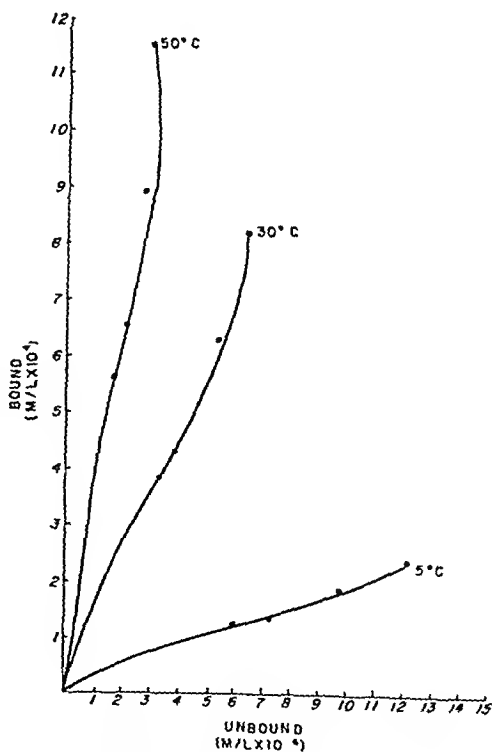


Fig. 4.—Binding of salicylic acid by nylon syringes.

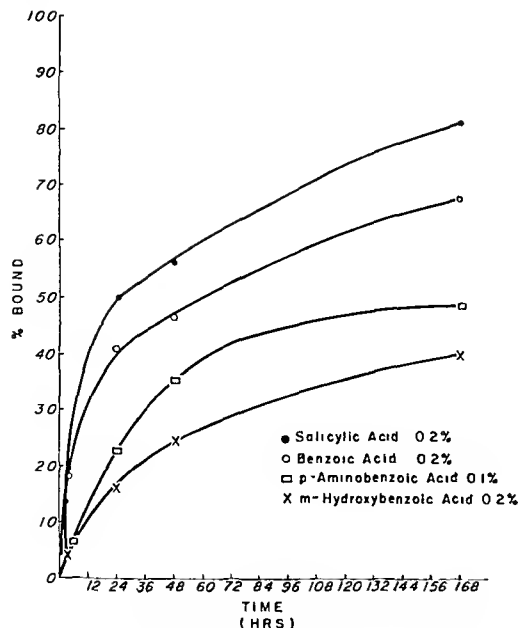


Fig. 5.—Binding of drugs by nylon syringes as a function of time.

concentration, temperature, and contact time were factors influencing the binding.

The magnitude of binding (quantity of drug or agent bound) shown in this paper and a previous paper indicates that diffusion is taking place, since the quantity of drug uptake cannot be attributed to surface binding alone. The number of sites on the surface exposed to the solution is relatively small compared to the quantity of molecules bound. It may be reasonably assumed that after the surface sites are filled, the solution permeates into the nylon where new sites for binding become available. Since no experiments were conducted to determine diffusion rates, quantitative interpretation of the diffusion phenomena could not be given. Certain results, however, gave strong evidence that the diffusion rates could be altered by the drug or agent, thus increasing or decreasing the total amount of drug bound.

In a previous report (5) the plot of bound agent *vs.* unbound agent resembled, to a first approximation, a Langmuir adsorption isotherm. Phenol and 4-chloro-3-methylphenol produced anomalous results which were explained on the basis that the two phenols were altering the structure of the macromolecule permitting a rapid surge of solution into the nylon when a certain critical concentration of the agent was reached. At the lower concentration range the two phenols mentioned above were taken up slowly until a critical concentration was reached where a dramatic uptake was noted.

In this study *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid (at 5°) followed the Langmuir adsorption isotherm when plotted as mentioned previously (Figs. 2, 3, and 4).

The plot for benzoic acid (Fig. 1) produced an S-shaped curve for each of the three temperatures. No definite explanation could be given for this type of curve, since sufficient data were not available for exact interpretation. The curve shows that the

uptake of the acid was not occurring in a smooth manner but rather in spurts as the concentration was increased. This type of curve suggests that in certain concentration ranges the benzoic acid had little difficulty reaching binding sites in the polymer. Once the polymer area in contact with the solution became saturated, no further binding could take place until the solution penetrated deeper into the plastic. When this diffusion took place a new series of sites became available for further binding.

The effect of temperature on binding is readily seen in Figs. 1–4, an increase in temperature causing greater binding. It is interesting to observe that within the forty-eight-hour period no binding occurred for *m*-hydroxybenzoic acid and very little binding for *p*-aminobenzoic acid at 5°. The increase in binding at the higher temperatures for all the drugs was assumed to be due to an increase in the diffusion rate of the solution into the plastic.

The one week's study (Fig. 5) indicates that significant quantities of the agents are bound even after short periods of time. Salicylic acid demonstrated the greatest degree of binding (Figs. 4 and 5) of the seven acidic agents included in the study. The esterification of the phenolic group in the salicylic acid (aspirin) obliterated this binding ability, probably because of the stereochemical effect.

The lack of binding of phenobarbital and barbital again might best be explained on steric hindrance preventing proper orientation of the acidic molecule to the sites in the macromolecule.

Results of the binding studies give further evidence that plastic materials should be thoroughly evaluated with the drug system to be employed or to be placed in contact with the plastic before routine use. The replacement of glass by a plastic material should necessitate careful considerations since glass has not shown the incompatibilities reported for plastic materials.

## SUMMARY

1. A previous study was continued to determine if three types of plastic syringes would bind seven weak organic acids.
2. Polyethylene and polystyrene barrels did not bind any of the seven agents.
3. The nylon barrel bound to various degrees, four of the seven agents (benzoic acid, *m*-hydroxybenzoic acid, *p*-aminobenzoic acid, and salicylic acid).
4. Acetylsalicylic acid, phenobarbital, and barbital were not bound by any of the plastic barrels.
5. The degree of binding was influenced by concentration, temperature, and diffusion.

## REFERENCES

- (1) Brewer, J. H., and Bryant, H. H., personal communication.
- (2) Autian, J., and Brewer, J. H., *Am. J. Hosp. Pharm.*, 15, 313(1958).
- (3) Autian, J., and Dhorda, C. N., *ibid.*, 16, 176(1959).
- (4) Autian, J., *Bull. Parenteral Drug Assoc.*, 12, 17(1958).
- (5) Marcus, E., Kim, H. K., and Autian, J., *This Journal*, 48, 457(1959).
- (6) Higuchi, T., and Lach, J. L., *ibid.*, 43, 465(1954).

# Studies on the Inhibitory Effect of Combined Chemical Preservatives on *Saccharomyces cerevisiae*\*

By H. G. OSMAN and AFAF EL-MARIAH

The minimal concentrations (Gm./L.) of formic, salicylic, benzoic, *o*- and *p*-chlorobenzoic acids, methyl-, ethyl-, propyl-, butyl esters of *p*-hydroxybenzoic acid, and sorbic acid required to inhibit the growth of *Saccharomyces cerevisiae* were determined. Combinations of any two of these chemicals were also tested for the same action. Salicylic acid with any of the other chemicals proved to have a potentiating effect. Other combinations had either an additive effect or even a lesser effect than their components used singly.

THE INHIBITORY EFFECT of many chemicals on the growth of microorganisms has been studied by many investigators. It was found that a chemical preservative may strongly inhibit the growth of certain microorganisms while having a lesser effect on others. Therefore, a mixture of two or more preservatives would have a wider scope of inhibition than their components when tested alone. This point was investigated by Sabalitschka (1), in 1932, who recommended the use of a mixture of 60 per cent ethyl *p*-hydroxybenzoate and 40 per cent propyl *p*-hydroxybenzoate for the protection of foodstuffs against deterioration through bacterial or fungal action.

Schellhorn (2), in 1950, noticed that in the presence of citric acid less benzoic acid was required to suppress the growth of microorganisms than when benzoic acid was used alone.

Littlejohn and Husa (3), in 1955, and Schimmel and Husa (4), in 1956, showed that there was either a definite potentiating action or an additive effect when mixtures of *p*-hydroxybenzoates were used as preservatives for syrups.

A further advantage of the combinations of *p*-hydroxybenzoic acid esters has been put forward by Boehm (5), in 1959, who found an increase in the solubilities of long-chain esters when they were combined together.

In this paper, some chemical preservatives were studied for their inhibitory effect on the growth of *Saccharomyces cerevisiae*. All possible combinations from two preservatives were prepared and their inhibitory effects were studied.

## EXPERIMENTAL

The test organism used throughout all these investigations was *Saccharomyces cerevisiae* which was kindly offered by Dr. K. Raible of the Deutsche Forschungsanstalt für Lebensmittelchemie. All chemicals used were of the extra pure or A. R. Merek grade.

*Saccharomyces cerevisiae* was cultured on a semi-

synthetic liquid medium containing, per L. (w/v): 30 Gm. sucrose; 8 Gm. citric acid; 3 Gm.  $\text{NH}_4\text{H}_2\text{PO}_4$ ; 8.9 Gm.  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ ; 0.1 Gm.  $\text{CaCl}_2$ ; 0.5 Gm.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 0.1 Gm. KCl; 1.5 Gm. Difco peptone; and 1.5 Gm. Difco yeast extract. The pH of the culture medium was always adjusted to 4.

The microorganism was subcultured on slopes of the same culture medium containing % (w/v) 2.5 Gm. agar agar. Transference of the yeast cells was carried out every month.

Sterilization of either the liquid culture medium or the slopes was always done at 5 lb./sq. in. for thirty minutes. The culture tubes or flasks were inoculated with a forty-eight-hour old liquid inoculum. The volume of the inoculum was always 2%. The cultures were incubated at 30° for one week. Unless otherwise specified, all experiments were carried out in 20-ml. test tubes containing 5 ml. culture medium.

For the determination of the growth of the yeast cultures the method adopted by Goodwin and Osman (6), in 1953, was applied. The relation between the absorbance at 1,000 m $\mu$  and the dry weight of the yeast cells has been studied. It was found that there was a linear relationship between the dry weight contents of the yeast cells in the liquid culture media and their corresponding extinctions at 1  $\mu$  over a wide range of *E* values. Figure 1 illustrates this relationship.

The *E* values were measured at 1  $\mu$  in order to exclude any absorption of light which might be due to any coloration of the liquid medium. Actively growing cultures were diluted beforehand with the liquid culture medium in order to obtain suitable readings on the Zeiss spectrophotometer model PMQ II.

## RESULTS AND DISCUSSION

In order to determine the period of incubation for the yeast cultures, the rate of growth was measured daily over a period of one week. Flasks, containing equal amounts of the liquid culture medium, were inoculated with equal volumes (2% inoculum) of an actively growing forty-eight-hour old inoculum. The absorbance of the liquid cultures was determined every twenty-four hours and the dry weight was calculated from the calibration curve. It was found that maximum growth was attained at about the third day of incubation as can be noticed from Table I.

\* Received August 12, 1959, from the National Research Centre, Dokki, Cairo, Egypt, U. A. R.



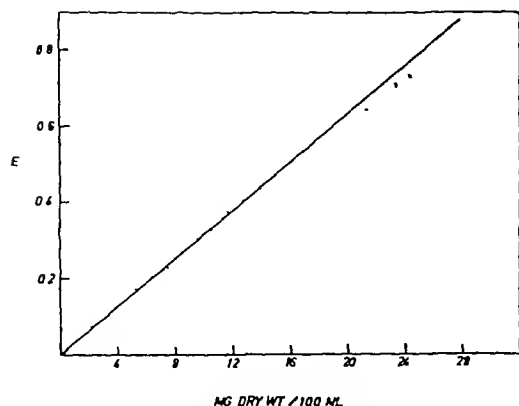


Fig. 1.—The calibration curve for converting  $E$  (1 cm., 1  $\mu$ ) of a suspension of *Saccharomyces cerevisiae* into dry weight values.

TABLE I.—RATE OF GROWTH OF *S. cerevisiae* GROWN ON A LIQUID CULTURE MEDIUM

Age of Culture, days	Dry Wt., mg./100 ml.	Age of Culture, days	Dry Wt., mg./100 ml.
1	7 52	5	125 70
2	95.14	6	
3	133 70	7	130 18
4	129 70	8	126 02

It was thought that a safe period of seven days was adequate for the yeast cultures to attain full growth.

**Inhibitory Effect of Preservatives When Tested Singly.**—The following chemicals, namely formic (I), salicylic (II), benzoic (III), *o*-chlorobenzoic (IV), *p*-chlorobenzoic (V) acids, methyl-(VI), ethyl-(VII), propyl-(VIII), and butyl-(IX) esters of *p*-hydroxybenzoic acid, sorbic acid (X), sodium sulfite (XI), and metabisulfite (XII) were studied singly for their inhibitory effect on the growth of *Saccharomyces cerevisiae* cultures.

All the preservatives, except salicylic acid, formic acid, sodium sulfite, and metabisulfite, were prepared as alcoholic solutions and the alcohol was removed by evaporation under reduced pressure. The others were added directly to the culture medium. Preliminary experiments were done by serial dilutions in order to determine, approximately, the minimal concentration at which complete suppression of growth takes place. Thereafter, narrower dilution ranges from each of the aforementioned preservatives were prepared and tested for their inhibitory effect. When the amount of growth was plotted against the concentration of the preservative, the exact concentration at which complete inhibition takes place, could be elucidated by extrapolating the line.

Table II shows the minimal concentration from each preservative which is required to completely inhibit the growth of *Saccharomyces cerevisiae*.

From Table II, it appears that the inhibitory effect increases from formic to *p*-chlorobenzoic acid. Suppression of growth by *p*-chlorobenzoic acid was much greater than *o*-chlorobenzoic acid. Furthermore, the activity increases progressively from the methyl- to the butyl ester of *p*-hydroxybenzoic acid

TABLE II.—THE MINIMAL CONCENTRATION OF CERTAIN CHEMICALS AT WHICH COMPLETE INHIBITION OF *Saccharomyces cerevisiae* TAKES PLACE

Chemical Preservatives	Minimal Concentration Required to Inhibit Growth Completely, Gm./L.
Formic acid (I)	3.389
Salicylic acid (II)	1.266
Benzoic acid (III)	0.358
<i>o</i> -Chlorobenzoic acid (IV)	1.550
<i>p</i> -Chlorobenzoic acid (V)	0.069
Methyl ester of <i>p</i> -hydroxybenzoic acid (VI)	1.117
Ethyl ester of <i>p</i> -hydroxybenzoic acid (VII)	0.629
Propyl ester of <i>p</i> -hydroxybenzoic acid (VIII)	0.234
Butyl ester of <i>p</i> -hydroxybenzoic acid (IX)	0.101
Sorbic acid (X)	0.370
Sodium sulfite (XI)	1 250
Sodium metabisulfite (XII)	1 250

and these results are in complete agreement with those obtained by Sabalitschka in 1929 (7), Neidig and Burrell in 1944 (8), as well as by Barkley in 1959 (9). In the case of sodium sulfite or metabisulfite, erratic results were obtained and an accurate determination of the minimal inhibitory concentration was not possible. Therefore, these two chemicals were excluded from the experiments carried out with combined preservatives.

**Inhibitory Effect of Combined Chemical Preservatives.**—The previously mentioned chemicals were examined in combinations of two in order to study their inhibitory effects. In these experiments, half the required minimal concentration of one chemical plus varying concentrations of the other were prepared and tested for their suppressing effect on the growth of *Saccharomyces cerevisiae*. These varying concentrations ranged from 25, 50, 75, and 100% of the minimal concentration.

Out of the 10 chemicals which were tested, it was possible to prepare 90 binary combinations. The results obtained are tabulated in Table III.

From Table III it appears that salicylic acid exerts a sparing effect on the amounts required from any of the other preservatives when combined with it. As an example, the combination of formic and salicylic acids is discussed in detail: when 50% of the required concentration from salicylic acid (0.633 Gm./L.) plus varying concentrations from formic acid were used, only 0.848 Gm./L. formic (25% of the minimal concentration) was needed instead of 1.695 Gm./L. (50%) which should have been present if the effect of the combinations were only additive. Thus, a saving of at least 25% of formic acid (0.848 Gm./L.) was achieved and this combination proved to have a potentizing action. If the case was reversed, i. e., 50% of the required formic acid (1.695 Gm./L.) plus varying concentrations from salicylic acid were combined, 0.633 Gm./L. salicylic 50% was required in order to inhibit the growth completely. In the latter case the effect of the combination was only additive.

The calculation of these effects can be done in another way similar to that employed by Littlejohn and Husa (1955).

TABLE III.—THE INHIBITORY ACTION OF COMBINED BINARY MIXTURES OF CHEMICAL PRESERVATIVES<sup>a</sup>

Preservative With Fixed Concn. 50%	Preservative With Variable Concn., %									
	I	II	III	IV	V	VI	VII	VIII	IX	X
I	...	≤50	≤75	≤50	≤75	≤50	≤50	≤75	≤75	≤100
II	≤25	...	≤25	≤25	≤25	≤25	≤25	≤25	≤25	≤37.5
III	≤50	≤50	...	≤75	≤75	≤75	≤75	≤100	≤100	≤100
IV	≤50	≤50	≤50	...	≤50	≤75	≤75	≤100	≤100	≤75
V	≤50	≤50	≤75	≤50	...	≤75	≤75	≤75	≤75	≤75
VI	≤50	≤50	≤75	≤50	≤50	...	≤50	≤50	≤75	≤75
VII	≤50	≤50	≤100	≤75	≤75	≤50	...	≤50	≤50	≤50
VIII	≤50	≤50	≤100	≤75	≤75	≤50	≤50	...	≤50	≤75
IX	≤75	≤50	≤100	≤100	≤75	≤75	≤75	≤50	...	≤75
X	≤100	≤50	≤100	≤75	≤75	≤75	≤75	≤75	≤75	...

<sup>a</sup> 100% from each preservative is the lowest concentration at which complete inhibition takes place.

According to our results, 0.633 Gm. salicylic plus 0.848 Gm. formic acid should theoretically preserve 750 ml. of the culture medium. Experimentally, it was found that 0.633 Gm. salicylic plus 0.848 Gm. formic acid actually preserves 1,000 ml. culture medium. So the potentizing effect is equal to  $[(1,000 \times 100)/750] - 100 = 33.3\%$ . In the case of sorbic acid, the potentizing effect would be  $[(1,000 \times 100)/875] - 100 = 14.3\%$ .

In the same way the decrease in the effectiveness of some preservatives when combined can be calculated on the same lines. As an example, 1.695 Gm. formic plus 0.2685 Gm. of benzoic acid should preserve 1,250 ml. culture but experimentally, it was found that this mixture preserved only 1,000 ml. culture medium. Thus the decrease in the activity through combination =  $[(1,000 \times 100)/1,250] - 100 = -20\%$ . In other examples where the combination was even less effective than the previous one, the combination of formic (50%) and sorbic acid is discussed. Formic, 1.695 Gm. plus 0.370 Gm. sorbic acid should preserve, 1,500 ml. culture medium but, by experiment, it was found that it preserved only 1,000 ml. So the decrease in the activity through combination =  $[(1,000 \times 100)/1,500] - 100 = -33.4\%$ . It can be concluded that combination of salicylic (50%) with any of the following preservatives, formic, benzoic, *o*-chlorobenzoic, *p*-chlorobenzoic acid, methyl-ethyl, propyl-, and butyl esters of *p*-hydroxybenzoic acid shows a potentizing effect of at least 33.3%; with sorbic acid it was only 14.3%.

Other combinations ( $\leq 50$ ) showed an additive or, probably, a slight potentizing effect which has not been determined. Table III also contains other combinations ( $\leq 75$ ,  $\leq 100$ ) which had an inhibitory effect less than the additive one. Here the preservative action was sometimes 80% and in some cases 86.6% of the additive value. In other words, higher concentrations of these preservatives were required in order to attain complete inhibition.

## SUMMARY

1. A method was devised for the spectrophotometric determination of the amount and rate of growth of *Saccharomyces cerevisiae* cultured in a semisynthetic liquid culture medium of pH 4.

2. The concentrations (Gm./L.) of formic, salicylic, benzoic, *o*-chloro- and *p*-chlorobenzoic acids, methyl-, ethyl-, propyl-, butyl esters of *p*-hydroxybenzoic acid, and sorbic acid required to inhibit the growth of the yeast was determined.

3. Combinations of half the required concentration of one preservative plus varying concentrations of the other were also tested for their suppressive action on the growth.

4. Combinations containing half the required concentration of salicylic acid plus any of the other preservatives proved to be more inhibitory than their components when tested singly.

5. Other combinations had a merely additive or even a less inhibitory action than their components.

## REFERENCES

- (1) Sabalitschka, T., *Mitt. Ges. Vorratsschutz*, **8**, 6(1932); cited by Littlejohn, O. M., and Husa, W. J., *THIS JOURNAL*, **44**, 305(1955).
- (2) Schelhorn, M. V., *Deut. Lebensm. Rundschau*, **46**, 132(1950).
- (3) Littlejohn, O. M., and Husa, W. J., *THIS JOURNAL*, **44**, 305(1955).
- (4) Schimmel, J., and Husa, W. J., *ibid.*, **45**, 204(1956).
- (5) Boehm, E., *Am. Perfumer Aromat.*, **73**, 37(1959).
- (6) Goodwin, T. W., and Osman, H. G., *Biochem. J.*, **53**, 541(1953).
- (7) Sabalitschka, T. H., *Z. angew. Chem.*, **42**, 936(1929).
- (8) Neidig, C. P., Burrell, H., *Drug. & Cosmetic Ind.*, **54**, 408, 481(1944).
- (9) Barkley, E. L., *Am. Perfumer Aromat.*, **73**, 33(1959).

# Influence of Hydrophil-Lipophil Balance on Ointment Bases\*

By JOHN W. RHYNE†, WILLIAM J. PAYNE, and CHARLES W. HARTMAN

A study of the effect of the hydrophil-lipophil balance (HLB) of ointment bases on the diffusion of water-soluble and water-insoluble drugs from the bases has been made. The diffusion of the drugs from bases having varying HLB values was determined by measuring the degree of inhibition of *Staphylococcus aureus* on serum agar plates. The drug diffusion as measured by inhibition varied considerably with change in the HLB value of the base. Phenylethyl alcohol, tyrothricin, and gramicidin gave no inhibition of *Staphylococcus aureus* in the concentrations used in the bases tested. The release of erythromycin, chlortetracycline HCl, neomycin sulfate, bacitracin, and hexachlorophene from ointment bases varied considerably with a change in the HLB value of the base.

THE HYDROPHIL-LIPOPHIL BALANCE system of classifying surface-active agents has found acceptance in pharmaceutical research relating to its applicability in the formulation of emulsions. Here it is applied to ointments to determine its effect upon drug release.

The purpose of this paper is to study the effect of the hydrophil-lipophil balance (HLB) of the base and the solubility of the medicament on drug release.

Reddish and Wales (1) conducted extensive tests on supposedly reliable antiseptic ointments of the U. S. P. X and the N. F. V and were confronted with the fact that only six of twelve U. S. P. ointments and only four of fourteen N. F. ointments showed any antiseptic activity whatever against *Staphylococcus aureus* on serum agar plates. Even phenol ointment U. S. P., containing the antiseptic upon which was based the phenol coefficient system for evaluating the efficacy of antiseptics, was found to possess negligible activity. This indicated that the type of base employed had some definite influence over release of the medicament, and subsequent research such as that of Johnston and Lee (2) has usually substantiated this belief.

**Effects of Surface-Active Agents in Ointments.**—The incorporation of emulsifying agents into ointment bases has been shown to have definite influence on their therapeutic performance (3, 4) and, generally speaking, this influence has been to improve the bases with respect to release of the drug and to

improve the pharmaceutical elegance of the product.

**The HLB System.**—According to Griffin (5) the HLB value is a numerical representation of the size and strength of the hydrophilic and lipophilic groups which form the molecule of a substance. In general, the lower the HLB value of a substance, the more lipophilic is that substance; whereas with increasing HLB values there exist increasing hydrophilic properties.

The authors believe that there should be an optimum HLB at which the diffusion of the medicament from an ointment base is most efficacious since emulsifying agents may make the ointment bases more miscible with the body fluids, thereby exposing more surface area of the ointment and allowing for more effective diffusion. By a theoretical extension of the HLB system, it should be possible to approximate the HLB of all the components of a base, including the therapeutically-active ingredients themselves, according to their hydrophilic and lipophilic tendencies and their solubility in water or organic solvents. Then one would be able to formulate an ointment that has an HLB value nearer the ideal with regard to release of the drug. A variation of the HLB within an ointment should give a corresponding variation in the release of medicament from that ointment. Upon inunction, it is assumed that at least part of the ointment is emulsified with tissue fluids. For different HLB values the type of emulsion and the particle size should be different.

To effect a practical application of this theory of HLB influence on drug release from ointments, some simple procedure for determining the HLB value of the ingredients (and the effect of the value of the HLB of the ointment) is needed. Some work in this area has been done by Chun, *et al.* (6), to determine the approximate HLB

\* Received from the University of Georgia, School of Pharmacy, Athens.

Abstracted in part from a thesis presented to the Graduate School, University of Georgia, in partial fulfillment of the requirements for the degree of Master of Science.

Adapted from the manuscript submitted by John W. Rhyme which received honorable mention in the 1958 Lunsford Richardson Pharmacy Awards competition.

† Present Address: Alcon Laboratories, Fort Worth, Texas.

Presented to the Scientific Section, A. P. A., Cincinnati meeting, August 1959.

of various natural emulsifying agents when in the presence of surface-active agents of known HLB values. Their method can be adapted to determine the HLB of other substances as well.

By using the equation of Chun, *et al* (6), in combination with the method of Griffin (5), the HLB value of an ointment formula, including all the constituents, might be determined and if the most effective HLB value for release of the particular drug were known, the HLB could then be adjusted by addition of suitable surface-active agents of known HLB to give an ointment base with an HLB value suitable for optimum drug release.

It is believed that drugs with different distribution coefficients in ointments with different HLB values would show a variation in drug release from the bases.

An attempt will be made to ascertain how a variation in HLB of the base will affect the diffusion of a drug from the base. In the future this may serve as a guide for the selection of a base with a definite HLB value which will give optimum drug release from an ointment.

## EXPERIMENTAL

**Determination of Drug Release.**—A modification of the agar plate method of the Food and Drug Administration (7) as described by Plavco and Husa (8) was employed.

**Preparation of Ointments.**—The primary base used in all ointments was white petrolatum U S P. Using this base, a series of HLB bases was prepared using emulsifying agents with HLB values varying from 1.8 to 17.9. Ten per cent, by weight, of surface-active agents of the polyoxyethylene, polyoxyethylene sorbitan, sorbitan series, having established HLB values as shown in Table I, were incorporated. The consistency of these bases shown in Table I, was checked by the U S P XV (9) method using a Precision Penetrometer (serial number 1-4, manufactured by Precision Scientific Co., Chicago, Ill.). It was desired to have the consistencies of the bases fall within or very near the range of consistency allowed for petrolatum, U S P.

Inhibition studies were carried out to determine if the variation in consistency of the base would cause a difference in the degree of inhibition. No significant difference in inhibition in the range of consistencies used was observed.

All of the drugs were incorporated into the bases by levigation. No levigating or solubilizing agents were used.

Drugs having different solubilities in water were chosen. Thus erythromycin, hexachlorophene, tyrothricin, and gramicidin are insoluble, whereas chlortetracycline HCl, neomycin sulfate, and phenylethyl alcohol are soluble in water. The drugs were incorporated into the bases on a weight in weight per cent basis as follows: erythromycin 1.0, chlortetracycline HCl 3.0, neomycin sulfate 0.6,

TABLE I—OINTMENT BASES, COMPOSITION CONSISTENCY AT 26° C, AND HLB VALUE

Surface-Active Agents Used <sup>a</sup>	Lot No	HLB <sup>c</sup>	Consistency of Penetration, mm <sup>d</sup>
Sorbitan trioleate	5241A	1.8	271.4
Sorbitan sesquioleate	6404C	3.7	270.1
Sorbitan monostearate	6539C	4.7	241.0
Sorbitan monopalmitate	32F	6.7	265.6
Sorbitan monolaurate	109	8.6	285.4
Polyoxyethylene sorbitan monostearate	185	9.6	279.6
Polyoxyethylene sorbitan monooleate	355	10.0	271.0
Polyoxyethylene sorbitan trioleate	297	11.0	285.4
Polyoxyethylene sorbitan monolaurate	6508B	13.3	272.7
Polyoxyethylene sorbitan monooleate	451	15.0	280.3
Polyoxyethylene sorbitan monopalmitate	185	15.6	287.6
Polyoxyethylene lauryl ether	219	16.7	278.0
Polyoxyethylene stearate	5725B	17.9	253.7

<sup>a</sup> All ointment bases consisted of 90% petrolatum U S P and 10% surface active agents.

<sup>b</sup> Products furnished by the Atlas Powder Co., Wilmington, Del.

<sup>c</sup> The HLB value is that assigned by Atlas Powder Co.

<sup>d</sup> All consistencies represent the average value obtained by the U S P XV method.

hexachlorophene 0.45, tyrothricin 0.1, gramicidin 0.05, and phenylethyl alcohol 5.0.

**Controls.**—Controls consisted of bases of identical HLB values with no medicament, petrolatum alone, and petrolatum with medicament. All controls were treated in the same manner as the HLB ointments containing medicaments.

All ointments were irradiated by ultraviolet light during the manufacturing process and then placed in ointment tubes which had also been irradiated to effect a state of near-sterility.

## RESULTS

**General.**—Phenylethyl alcohol, tyrothricin, and gramicidin used in therapeutic concentrations equivalent to those commonly used in commercial ointments gave no significant inhibition of *Staphylococcus aureus* at the HLB values tested in these experiments.

**Neomycin Sulfate.**—As seen in Fig. 1, neomycin sulfate in all three trials gave better inhibition in the ointments with HLB values between 8 and 16. Therefore, neomycin sulfate must have diffused more readily between the HLB value of 8 and 16.

**Chlortetracycline Hydrochloride.**—As shown in Fig. 1, chlortetracycline hydrochloride gave greater inhibition in ointments with high HLB values.

**Erythromycin.**—As seen in Fig. 2, the erythromycin ointments with low HLB values gave greater inhibition.

**Hexachlorophene.**—Figure 2 shows that for hexachlorophene the inhibition is almost constant except for two apparent peaks at HLB 8.6 and HLB 10.0, with the least inhibition found at HLB 9.6 and 17.9.

All drugs showed a decrease in inhibition at HLB 17.9.

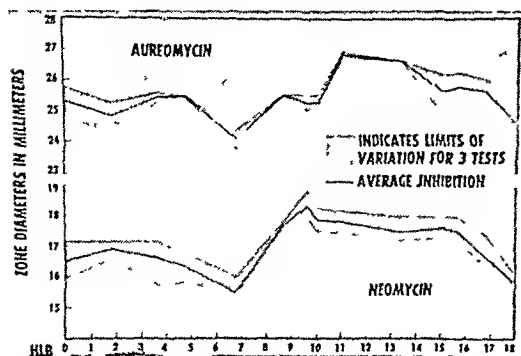


Fig 1—Inhibition of *Staphylococcus aureus* by Aureomycin hydrochloride and neomycin sulfate ointments with varying HLB values

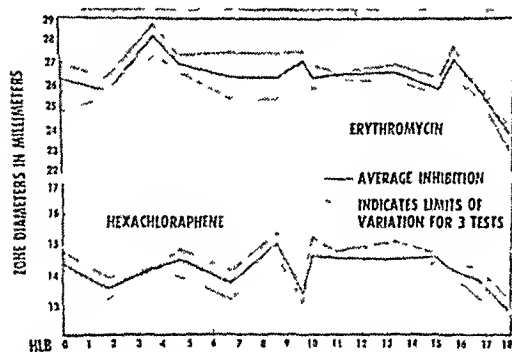


Fig 2—Inhibition of *Staphylococcus aureus* by erythromycin and hexachlorophene ointments with varying HLB values

Controls.—No inhibition was observed for the control ointments containing petrolatum, U S P with the various surface-active agents

It was thought that the differences in inhibition as shown in these experiments might be due to ex-

perimental error, but it may be seen that in all three determinations for each drug the variations in inhibition are similar. Average curves were drawn using the three test results but variation did not differ significantly from the individual curves as shown by the upper and lower limits. It was also considered possible that the variation in inhibition might be due to variation in consistency, but experiments with varying consistencies did not serve to substantiate this. It is felt that factors other than HLB should be considered in further work. The distribution coefficient of the drug and the possibility of intermolecular activity may well play an important part in the diffusion of drugs from ointment bases

## CONCLUSIONS

The HLB of an ointment base has some influence on the diffusion of the medicament from that base. From this work it may be surmised that the HLB value of an ointment base should be considered when attempting to adjust that base to increase or decrease the rate of drug release. Insufficient data does not warrant a conclusion concerning the effect of the solubility of the drug on release from bases having different HLB values.

## REFERENCES

- (1) Reddish, G F, and Wales, H J, *THIS JOURNAL*, 18, 576(1929)
- (2) Johnston, G W, and Lee, C O, *ibid*, 32, 278(1943)
- (3) McDonald, L H, and Himechek, R E, *ibid*, 37, 368(1948)
- (4) Dodd, M C, Hartmann, F W, and Ward, W C, *ibid*, 35, 33(1946)
- (5) Griffin, W C, *J Soc Cosmetic Chemists*, 1, 311(1949)
- (6) Chun, A H C, Joslin, R S, and Martin, A N, *Drug & Cosmetic Ind*, 82, 164(1958)
- (7) "The Compilation of Regulations for Tests and Methods of Assay for Antibiotic Drugs," U S Dept of Health, Education, and Welfare, Food and Drug Administration April 1951 Sec 141.401 and 141.402
- (8) Plaxco, J M, Jr, and Husa, W J, *THIS JOURNAL*, 45, 141(1956)
- (9) "U S Pharmacopeia," 15th Rev, Mack Publishing Co, Easton, Pa, 1955, p 930

# The Interaction of Citrate With Aspirin and Benzoic Acid\*

By SANFORD BOLTON

Solubility studies were used to determine the nature and extent of the interaction between sodium citrate and aspirin. Because of the complex nature of this system, parallel studies of the solubilizing action of sodium citrate and acetate on benzoic acid were made for purposes of comparison. The acetate-benzoic acid reaction closely followed results expected from mass law considerations over a wide concentration range. However, the weak acid-citrate interactions could be described by the mass law only at low citrate concentrations, showing wide deviations at higher concentrations. It was apparent that the deviations were a function of many variables which included changes of solubility and  $K_a$  with the nature and concentration of the solution species. The fact that the interactions could not be rationalized by these changes alone at very high citrate concentrations suggested an association or associations among the dissolved species.

It has been long recognized that aspirin can be solubilized in aqueous solutions of citrate salts. Such combinations have been shown to produce more stable solutions of aspirin than those obtained when solubilization is effected by other bases (1). Although the increase of aspirin stability in these systems has been suggested for some time, no specific investigation which could explain this phenomenon has been reported. It is the purpose of the present study to attempt such an investigation.

The experimental approach consisted of noting changes in the solubility and pH of saturated solutions of aspirin in the presence of varying amounts of citrate and strong base. The observed results were to be compared with results expected theoretically. Possible association of species in this system, which could explain the increased stability, would be indicated by profound deviations from expected results. Because this type of interaction is very complex, parallel studies were made with benzoic acid.<sup>1</sup> This acid was chosen because of its structural similarity to aspirin and because of the large quantity of data available on the effect of environment on its activity in aqueous solutions. Certain concurrent studies were made in order to help elucidate the above reactions. These included (a) the effect of citric acid on the solubilities of undissociated aspirin and benzoic acid, (b) salt effects, and (c) the effect of concentration on the  $K_a$  values of citric acid.

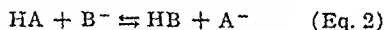
## THEORY

A paper by Higuchi, Gupta, and Busse (3) has described equations for the calculation of the solubility of difficultly soluble, weakly acidic drugs as a function of pH. Equation 1 was found to be approximately valid for a variety of weak acids in solutions of constant ionic strength when  $S$  and  $S_0$  were expressed as concentrations rather than activities.

$$\log (S - S_0) = \log S_0 - pK_a + pH \quad (\text{Eq. 1})$$

$S$  = Total solubility and  $S_0$  = solubility of undissociated acid.

The interaction of a weak acid and weak base may be represented by the following equations:



$$K = K_a/K_b = (HB)(A^-)/(HA)(B^-) \quad (\text{Eq. 3})$$

where

$$K_a = (A^-)(H^+)/(HA) \quad (\text{Eq. 4})$$

and

$$K_b = (B^-)(H^+)/(HB) \quad (\text{Eq. 5})$$

If  $HA$  represents a slightly soluble acid and if the system is saturated with this acid, Eq. 3 may be expressed as

$$K_a(S_0)/K_b = (HB)(A^-)/(B^-) \quad (\text{Eq. 6})$$

As is the case in Eq. 1, this relationship is valid only when concentrations are replaced by activities. In dilute solutions, activity generally approximates concentration; but in concentrated solutions, deviations from Eqs. 1 and 6 are to be expected. The magnitude and direction of such deviations are dependent, generally, on the nature of the solution species.

## EXPERIMENTAL

**Reagents.**—Recrystallized aspirin, m. p. 133°; benzoic acid, m. p. 122°; sodium acetate, anhydrous; sodium chloride, anhydrous; sodium citrate, dihydrate U. S. P.; citric acid, monohydrate U. S. P.; sodium hydroxide solution, carbonate free.

\* Received August 21, 1959, from the College of Pharmacy, University of Rhode Island, Kingston.  
Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.

<sup>1</sup> Doosar and Bhagwat (2) had followed the interaction of benzoic acid with citrate and acetate at low concentrations in order to determine dissociation constants. In the present study, more complete data are presented at both low and high salt concentrations.

**Procedure.**—An excess of aspirin or benzoic acid was shaken with 10 ml of solution containing varying amounts of the appropriate reagents in a constant temperature water bath at 30°. The aspirin solutions were shaken for three hours to keep hydrolysis to a minimum. The benzoic acid solutions were shaken for twelve hours. The pH of each solution, then, was determined by use of a Beckman model G pH meter, and clear aliquots were diluted with 0.02 *N* hydrochloric acid for spectrophotometric analysis. In the salting out studies and in the studies concerned with the effect of citric acid on the solubilities of benzoic acid and aspirin, 0.02 *N* hydrochloric acid was added to keep dissociation of the acids to a minimum.

**Analysis.** The peak at 273 *mμ* in the U. V. spectra was used as a basis for the analysis of benzoic acid. In the aspirin studies, the amount of salicylic acid formed was determined by its peak at 302.5 *mμ*. The minimum in the U. V. spectrum at 259 *mμ* was then used to determine the amount of aspirin in solution.

## RESULTS AND DISCUSSION

**Citric Acid-Aspirin and Citric Acid-Benzoic Acid Interactions.**—The effect of citric acid on the solubilities of undissociated aspirin and benzoic acid is shown in Fig. 1. The apparent interaction between these uncharged species is slight. The results, however, suggest the possibility of associations in solutions of higher pH where charged basic moieties may exist. Kolthoff and Bosch (4) and Larsson (5) have noted the presence of this type of complex in the benzoic acid-benzoate system.

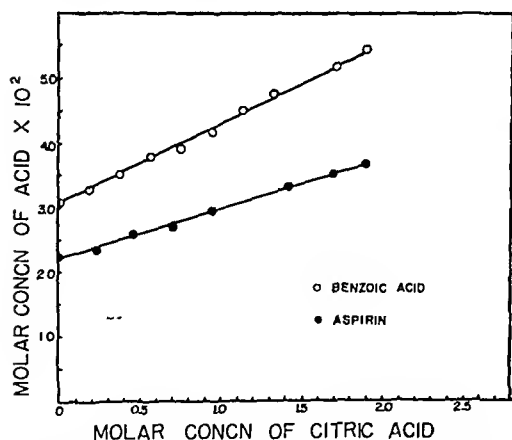


Fig. 1.—The effect of citric acid on the solubilities of undissociated benzoic acid and aspirin at 30°.

**Salt Effects.**—The extent of interaction in the weak base-insoluble weak acid reactions depends on the values of  $K_a$ ,  $K_b$ , and  $S_0$  (Eq. 6). These values are greatly dependent on the nature of the ionic environment. It is virtually impossible to determine these effects in the systems investigated in this report. It appears that the best approach in an analysis of these systems would be to use the results of salt effects in relatively simple systems as an approximation of such effects in the present investigation.

The influence of sodium chloride on the solubilities of the weak acids is depicted in Table I and Fig. 2. In the case of benzoic acid, these results agree closely with those obtained by Kolthoff and Bosch (4) at 25°.

TABLE I—THE EFFECT OF SODIUM CHLORIDE ON THE SOLUBILITY OF BENZOIC ACID AND ASPIRIN AT 30°

Ionic Strength	Benzoic Acid Solubility, Moles/L	Ionic Strength	Aspirin Solubility, Moles/L
0	0.031	0	0.022
0.276	0.0271	0.171	0.0216
0.550	0.0239	0.430	0.0190
0.824	0.0211	0.514	0.0169
1.12	0.0189	0.600	0.0174
1.39	0.0171	0.684	0.0165
1.66	0.0159	0.770	0.0161
1.93	0.0141	0.855	0.0148
2.21	0.0125	1.03	0.0139
2.49	0.0112	1.20	0.0130
2.76	0.0102	1.37	0.0116
		1.54	0.0106
		1.71	0.0102
		1.90	0.0086
		2.06	0.0088
		2.40	0.0072
		2.74	0.0068
		3.08	0.0051
		3.42	0.0045

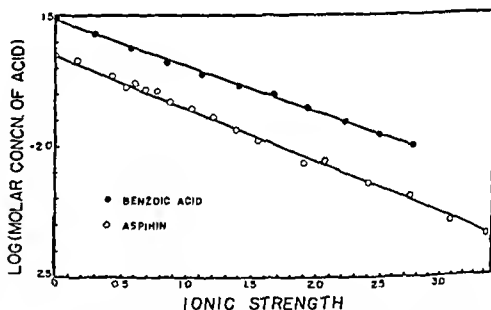


Fig. 2.—The effect of sodium chloride on the solubilities of undissociated benzoic acid and aspirin at 30°.

Although no studies of salt effects on  $K_a$  values were made in this study, a review of some pertinent data is included here. Riesch and Kilpatrick (6) and Guntelberg and Schrodt (7) have shown that the apparent  $K_a$  of benzoic acid increased with salt concentration to a maximum value of  $11.9 \times 10^{-5}$  at 0.5 *M* sodium chloride. At higher ionic strengths, the  $K_a$  gradually decreased to  $2.46 \times 10^{-5}$  at 5.0 *M* sodium chloride.

Several investigators have published data on the effect of foreign neutral salts (8, 9), as well as the effect of acetate (10) on the  $K_a$  of acetic acid. Again, a maximum in  $K_a$  was found to occur at approximately 0.5 *M* sodium chloride. In the presence of sodium acetate, similar results were obtained, the maximum being dependent both on the amount of acetate present and on the ratio of acetate to acetic acid. The original publications should be consulted for more detail.

**The Effect of Concentration on the Dissociation Constants of Citric Acid.**—Adell (11) has demonstrated that the apparent values of the  $K_a$ 's for citric acid increase in the presence of sodium chloride. Kolthoff and Bosch (12) showed that the  $K_a$ 's also increase with the concentration of citric acid and its corresponding potassium salts. Since their studies involved total citric acid concentrations which were comparatively small, an extension of these studies, with sodium citrate, to higher concentrations was made. The results are shown in Table II. The present work was not intended to yield exact results. However, the values do give a good approximation of variation of the apparent values of the  $K_a$ 's with respect to concentration. The  $K_a$ 's were determined by potentiometric titrations, utilizing the one-half neutralization point in the titration curves.<sup>2</sup>

TABLE II.—CONCENTRATION EFFECTS ON THE DISSOCIATION CONSTANTS OF CITRIC ACID

Molar Concn. of Citric Acid and Dihydrogen Citrate <sup>a</sup>	Apparent $pK_{a1}$
0.0115	3.03
0.091	2.97
0.232	2.86
0.455	2.79
0.910	2.55

Molar Concn. of Dihydrogen Citrate and Monohydrogen Citrate	Apparent $pK_{a2}$
0.011	4.55
0.083	4.34
0.221	4.19
0.415	4.12
0.835	3.91

Molar Concn. of Monohydrogen Citrate and Citrate	Apparent $pK_{a3}$
0.011	5.89
0.077	5.62
0.212	5.36
0.334	5.26
0.765	5.13

<sup>a</sup> A concentration of 0.0115 means that each species is present in approximately this concentration.

**Weak Acid-Base Interactions.**—The solubility of aspirin and benzoic acid as a function of pH in the presence of various bases is shown in Figs. 3 and 4 and Tables III and IV. (See also Tables V, VI, and VII.) The solid lines in Figs. 3 and 4 represent values calculated from Eq. 1

The results of the solubilization of the acids by sodium hydroxide conform closely to the calculated values, showing some deviation at high concentrations

The acetate-benzoic acid interaction also followed closely results expected from Eq. 1. It may be noted that the position of the straight lines of slope 1 in Figs. 3 and 4 is determined by the "y" intercept,  $\log S_0 - pK_a$ . Increased ionic environment, in the acetate-benzoic acid system results in a simultaneous decrease in the values of  $\log S_0$  and  $pK_a$  for benzoic acid. This compensatory effect would tend to fix the position of the straight line.

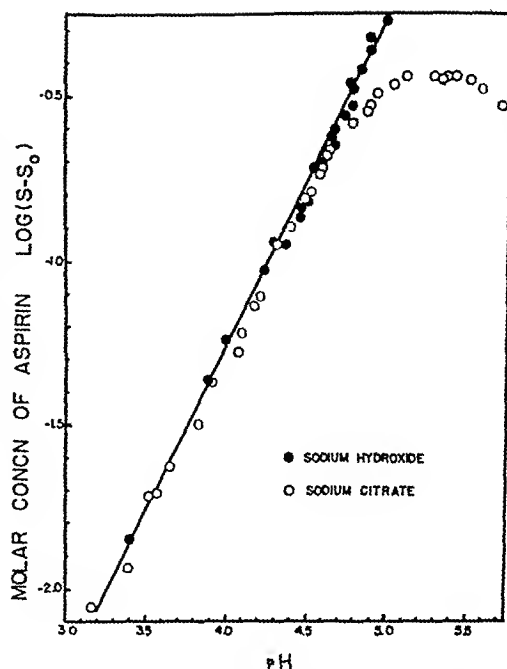


Fig. 3.—Influence of pH on the solubility of aspirin in the presence of sodium hydroxide and sodium citrate at 30°. The solid line represents values calculated from Eq. 1.

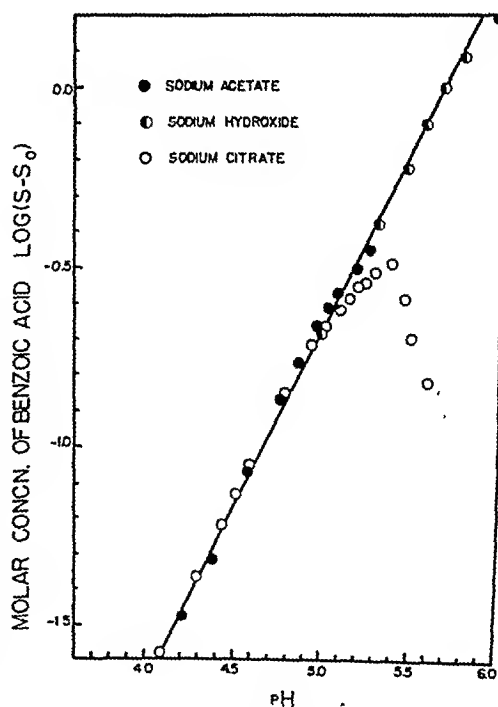


Fig. 4.—Influence of pH on the solubility of benzoic acid in the presence of sodium hydroxide, sodium citrate, and sodium acetate at 30°. The solid line represents values calculated from Eq. 1.

<sup>2</sup> Appropriate corrections for the effect of other ionization constants were made according to a method suggested by Kolthoff and Bosch (12).



TABLE III—SOLUBILITY OF ASPIRIN AS A FUNCTION OF pH AT 30°

Final pH	Solubility, Moles/L.
1 70 <sup>a</sup>	0 022
2 60 <sup>a</sup>	0 024
3 40	0 036
3 90	0 076
4 00	0 079
4 20 <sup>a</sup>	0 098
4 24	0 116
4 30	0 138
4 38	0 135
4 47	0 168
4 47	0 156
4 50 <sup>a</sup>	0 180
4 52	0 175
4 55	0 212
4 60	0 222
4 66	0 261
4 68	0 275
4 68	0 244
4 74	0 295
4 78	0 372
4 79	0 316
4 80	0 352
4 84	0 403
4 90	0 458
4 90	0 504
5 00	0 554
5 10 <sup>a</sup>	0 610
5 10 <sup>a</sup>	0 610

<sup>a</sup> These points were omitted from Fig 3 due to spatial limitations

TABLE IV—SOLUBILITY OF BENZOIC ACID AS A FUNCTION OF pH AT 30°

Final pH	Solubility, Moles/L
2 85 <sup>a</sup>	0 032
5 01	0 239
5 33	0 445
5 50	0 630
5 60	0 817
5 70	1 027
5 82	1 249
6 00	1 600
6 23 <sup>a</sup>	2 110
6 36 <sup>a</sup>	2 470

<sup>a</sup> These points were omitted from Fig 4 due to spatial limitations

Thus, the apparent agreement with Eq 1 in this case may be merely coincidental

In the cases of the citrate-weak acid systems, Eq 1 describes the interactions only at low citrate concentrations. The decrease in solubility at high citrate concentrations was not due to an insoluble complex as was shown by analysis of the solid phase in appropriate containers

Figures 5, 6, and 7 and Tables V, VI, and VII show the effect of citrate and acetate concentration on the solubilities of aspirin and benzoic acid. The observed results are compared with values calculated from Eq 6

With the assumption that the ionic species in solution exert effects similar to sodium chloride, the value of  $K_a(So)/K_b$  (Eq 6) can be approximated and the extent of interaction calculated. It is of interest to note that in the acetate-benzoic

TABLE V—SOLUBILITY OF ASPIRIN AS A FUNCTION OF SODIUM CITRATE CONCENTRATION AT 30°

Sodium Citrate, Moles/L	Aspirin Solubility, Moles/L	pH
0 0034	0 0306	3 16
0 0068	0 0332	3 39
0 0085	0 0407	3 51
0 0102	0 0412	3 57
0 0136	0 0453	3 65
0 0170	0 0533	3 83
0 0255	0 0646	3 91
0 0340	0 0746	4 08
0 0425	0 0830	4 10
0 0510	0 0954	4 18
0 0595	0 0993	4 21
0 0850	0 135	4 32
0 102	0 149	4 40
0 136	0 178	4 50
0 153	0 183	4 53
0 170	0 203	4 58
0 204	0 213	4 60
0 255	0 243	4 65
0 332	0 284	4 79
0 408	0 306	4 88
0 510	0 317	4 90
0 545	0 343	4 95
0 680	0 366	5 05
0 816	0 386	5 13
1 090	0 382	5 30
1 190	0 379	5 36
1 224	0 386	5 38
1 360	0 389	5 44
1 530	0 378	5 53
1 700	0 354	5 60
1 900	0 319	5 72

TABLE VI—SOLUBILITY OF BENZOIC ACID AS A FUNCTION OF SODIUM CITRATE CONCENTRATION AT 30°

Sodium Citrate, Moles/L	Benzoic Acid Solubility, Moles/L	pH
0 0136	0 0572	4 09
0 0272	0 0747	4 30
0 0408	0 0917	4 44
0 0544	0 105	4 52
0 068	0 120	4 60
0 136	0 171	4 80
0 204	0 223	4 94
0 272	0 246	5 04
0 340	0 270	5 11
0 408	0 288	5 17
0 476	0 309	5 21
0 544	0 316	5 26
0 680	0 332	5 33
0 850	0 351	5 41
1 190	0 288	5 49
1 360	0 231	5 52
1 700	0 180	5 61

acid system such calculations<sup>3</sup> yield a nearly constant value for  $K_a(So)/K_b$ , which might explain the near coincidence of the curves in Fig 7.

Similar considerations may be used to approximate the extent of interaction in the citrate-benzoic acid reaction. The value,  $K_a(So)/K_b$ , would be expected to decrease at high ionic strengths, since  $K_a$  and  $So$  for benzoic acid would decrease and  $K_b$ ,

<sup>3</sup> The data used for these calculations were taken from references (9) and (11) as well as from the present study

TABLE VII.—SOLUBILITY OF BENZOIC ACID AS A FUNCTION OF SODIUM ACETATE CONCENTRATION AT 30°

Sodium Acetate, Moles/L.	Benzoic Acid Solubility, Moles/L.	pH
0.0428	0.0645	4.22
0.0713	0.0792	4.39
0.143	0.115	4.59
0.285	0.166	4.78
0.429	0.203	4.88
0.570	0.246	4.98
0.713	0.275	5.05
0.858	0.295	5.10
1.140	0.344	5.21
1.430	0.383	5.28

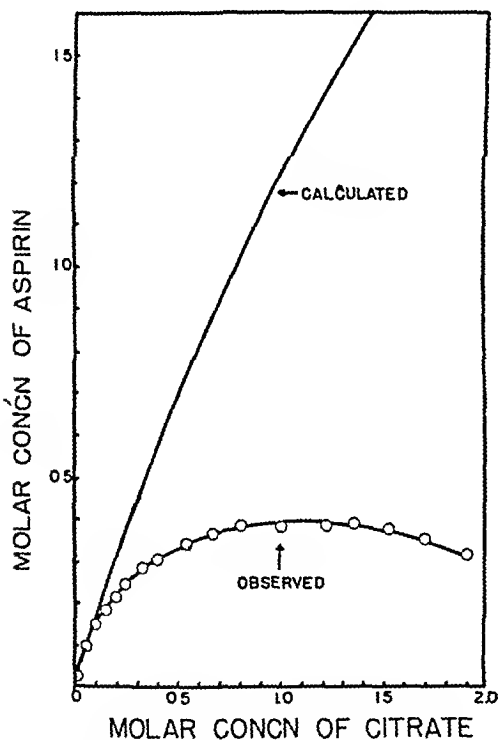


Fig 5—The effect of sodium citrate on the solubility of aspirin at 30°. Observed values are compared with those calculated from Eq 6

the third dissociation constant for citric acid,<sup>4</sup> would increase. The apparent decrease in the interaction at high citrate concentrations as observed in Figs 5 and 6 may be explained on this basis.

A further analysis of the experimental results in solutions of high citrate concentration can yield an approximate value for the third dissociation constant of citric acid. In the absence of interfering reactions, the amount of benzoate formed would be equal to the amount of monohydrogen citrate in solution. This fact, the knowledge of the amount of citrate added to the system, and the observed pH can be used to calculate the constant. The values so obtained revealed abnormally high dissociation constants at high citrate concentrations in the ben-

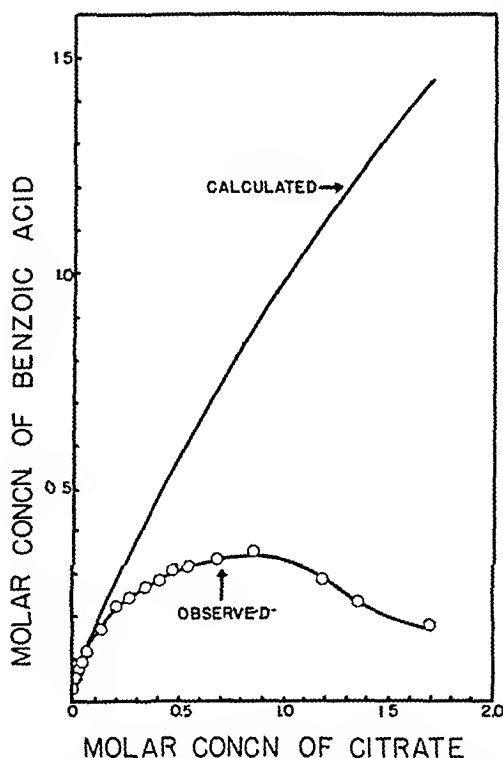


Fig 6—The effect of sodium citrate on the solubility of benzoic acid at 30°. Observed values are compared with those calculated from Eq 6

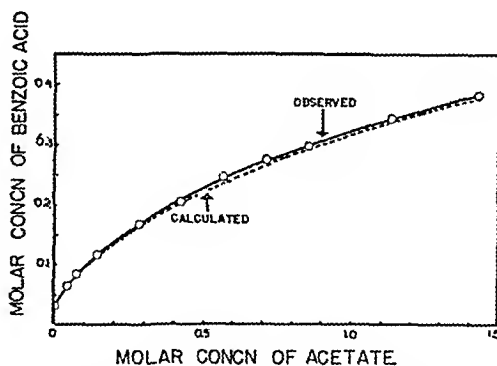


Fig 7—The effect of sodium acetate on the solubility of benzoic acid at 30°. Observed values are compared with those calculated from Eq 6

zoic acid interaction. For example, at 1.70 M citrate, the calculated value of  $K_{a3}$  was approximately  $2 \times 10^{-5}$ . If citrate ions were being used up in competing reactions, such as association with itself or other solvent species, the above observations could be readily accounted for.

It appears that similar considerations would apply to the citrate-aspirin system due to the similarity of the acids and the experimental results. Since a citrate complex cannot be actually demonstrated, especially at low concentrations, it is difficult to rationalize previous reports of increased stability of aspirin in citrate solutions.

<sup>4</sup> At high concentrations of citrate, the predominant reaction is benzoic acid + citrate<sup>3-</sup>  $\rightleftharpoons$  benzoate<sup>-</sup> + H citrate<sup>2-</sup>.

TABLE III—SOLUBILITY OF ASPIRIN AS A FUNCTION OF pH AT 30°

Final pH	Solubility, Moles/L.
1 70 <sup>a</sup>	0 022
2 60 <sup>a</sup>	0 024
3 40	0 036
3 90	0 076
4 00	0 079
4 20 <sup>a</sup>	0 098
4 24	0 116
4 30	0 138
4 38	0 135
4 47	0 168
4 47	0 156
4 50 <sup>a</sup>	0 180
4 52	0 175
4 55	0 212
4 60	0 222
4 66	0 261
4 68	0 275
4 68	0 244
4 74	0 295
4 78	0 372
4 79	0 316
4 80	0 352
4 84	0 403
4 90	0 458
4 90	0 504
5 00	0 554
5 10 <sup>a</sup>	0 610
5 10 <sup>a</sup>	0 610

<sup>a</sup> These points were omitted from Fig 3 due to spatial limitations

TABLE IV—SOLUBILITY OF BENZOIC ACID AS A FUNCTION OF pH AT 30°

Final pH	Solubility, Moles/L.
2 85 <sup>a</sup>	0 032
5 01	0 239
5 33	0 445
5 50	0 630
5 60	0 817
5 70	1 027
5 82	1 249
6 00	1 600
6 23 <sup>a</sup>	2 110
6 36 <sup>a</sup>	2 470

<sup>a</sup> These points were omitted from Fig 4 due to spatial limitations

Thus, the apparent agreement with Eq 1 in this case may be merely coincidental

In the cases of the citrate-weak acid systems, Eq 1 describes the interactions only at low citrate concentrations. The decrease in solubility at high citrate concentrations was not due to an insoluble complex as was shown by analysis of the solid phase in appropriate containers

Figures 5, 6, and 7 and Tables V, VI, and VII show the effect of citrate and acetate concentration on the solubilities of aspirin and benzoic acid. The observed results are compared with values calculated from Eq 6

With the assumption that the ionic species in solution exert effects similar to sodium chloride, the value of  $K_a(So)/K_b$  (Eq 6) can be approximated and the extent of interaction calculated. It is of interest to note that in the acetate-benzoic

TABLE V—SOLUBILITY OF ASPIRIN AS A FUNCTION OF SODIUM CITRATE CONCENTRATION AT 30°

Sodium Citrate, Moles/L.	Aspirin Solubility, Moles/L.	pH
0 0034	0 0306	3 16
0 0068	0 0332	3 39
0 0085	0 0407	3 51
0 0102	0 0412	3 57
0 0136	0 0453	3 65
0 0170	0 0533	3 83
0 0255	0 0646	3 91
0 0340	0 0746	4 08
0 0425	0 0830	4 10
0 0510	0 0954	4 18
0 0595	0 0993	4 21
0 0850	0 135	4 32
0 102	0 149	4 40
0 136	0 178	4 50
0 153	0 183	4 53
0 170	0 203	4 58
0 204	0 213	4 60
0 255	0 243	4 65
0 332	0 284	4 79
0 408	0 306	4 88
0 510	0 317	4 90
0 545	0 343	4 95
0 680	0 366	5 05
0 816	0 386	5 13
1 090	0 382	5 30
1 190	0 379	5 36
1 224	0 386	5 38
1 360	0 389	5 44
1 530	0 378	5 53
1 700	0 354	5 60
1 900	0 319	5 72

TABLE VI—SOLUBILITY OF BENZOIC ACID AS A FUNCTION OF SODIUM CITRATE CONCENTRATION AT 30°

Sodium Citrate, Moles/L.	Benzoic Acid Solubility, Moles/L.	pH
0 0136	0 0572	4 09
0 0272	0 0747	4 30
0 0408	0 0917	4 44
0 0544	0 105	4 52
0 068	0 120	4 60
0 136	0 171	4 80
0 204	0 223	4 94
0 272	0 246	5 04
0 340	0 270	5 11
0 408	0 288	5 17
0 476	0 309	5 21
0 544	0 316	5 26
0 680	0 332	5 33
0 850	0 351	5 41
1 190	0 288	5 49
1 360	0 231	5 52
1 700	0 180	5 61

acid system such calculations<sup>3</sup> yield a nearly constant value for  $K_a(So)/K_b$ , which might explain the near coincidence of the curves in Fig 7.

Similar considerations may be used to approximate the extent of interaction in the citrate-benzoic acid reaction. The value,  $K_a(So)/K_b$ , would be expected to decrease at high ionic strengths, since  $K_a$  and  $So$  for benzoic acid would decrease and  $K_b$ ,

<sup>3</sup> The data used for these calculations were taken from references (9) and (11) as well as from the present study

TABLE VII.—SOLUBILITY OF BENZOIC ACID AS A FUNCTION OF SODIUM ACETATE CONCENTRATION AT 30°

Sodium Acetate, Moles/L.	Benzoic Acid Solubility, Moles/L.	pH
0.0428	0.0645	4.22
0.0713	0.0792	4.39
0.143	0.115	4.59
0.285	0.166	4.78
0.429	0.203	4.88
0.570	0.246	4.98
0.713	0.275	5.05
0.858	0.295	5.10
1.140	0.344	5.21
1.430	0.383	5.28

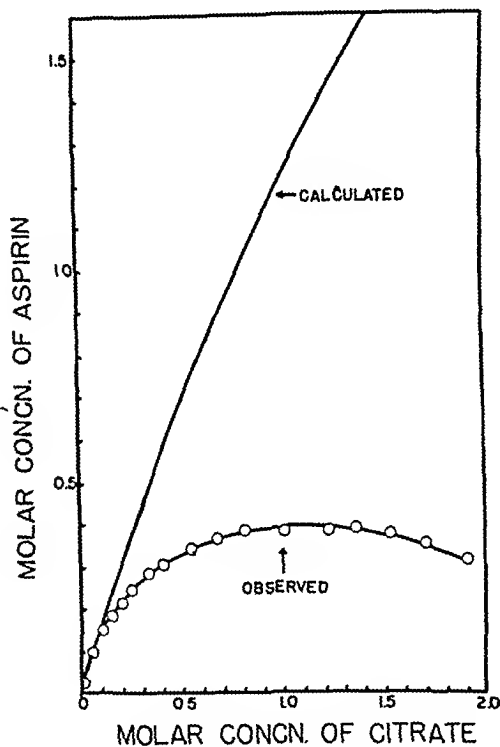


Fig. 5.—The effect of sodium citrate on the solubility of aspirin at 30°. Observed values are compared with those calculated from Eq. 6.

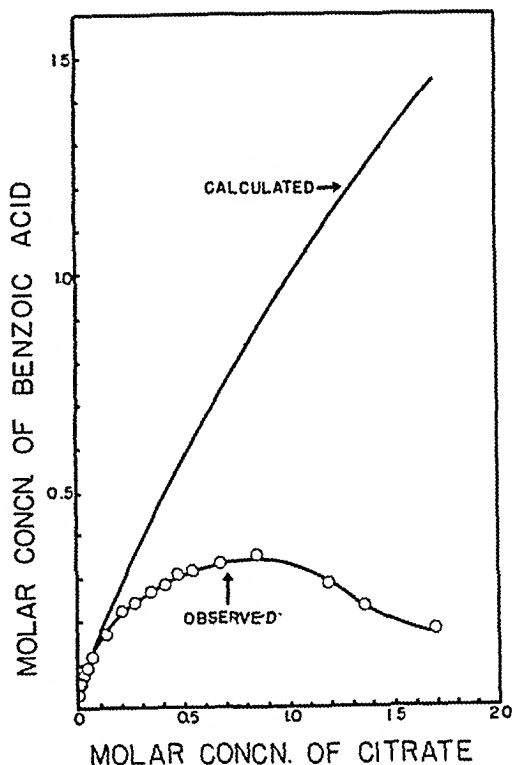


Fig. 6.—The effect of sodium citrate on the solubility of benzoic acid at 30°. Observed values are compared with those calculated from Eq. 6.

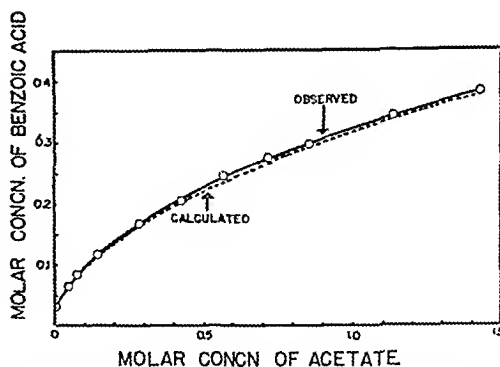


Fig. 7.—The effect of sodium acetate on the solubility of benzoic acid at 30°. Observed values are compared with those calculated from Eq. 6.

the third dissociation constant for citric acid,<sup>4</sup> would increase. The apparent decrease in the interaction at high citrate concentrations as observed in Figs. 5 and 6 may be explained on this basis.

A further analysis of the experimental results in solutions of high citrate concentration can yield an approximate value for the third dissociation constant of citric acid. In the absence of interfering reactions, the amount of benzoate formed would be equal to the amount of monohydrogen citrate in solution. This fact, the knowledge of the amount of citrate added to the system, and the observed pH can be used to calculate the constant. The values so obtained revealed abnormally high dissociation constants at high citrate concentrations in the ben-

zoic acid interaction. For example, at 1.70 *M* citrate, the calculated value of  $K_{a3}$  was approximately  $2 \times 10^{-3}$ . If citrate ions were being used up in competing reactions, such as association with itself or other solvent species, the above observations could be readily accounted for.

It appears that similar considerations would apply to the citrate-aspirin system due to the similarity of the acids and the experimental results. Since a citrate complex cannot be actually demonstrated, especially at low concentrations, it is difficult to rationalize previous reports of increased stability of aspirin in citrate solutions

<sup>4</sup> At high concentrations of citrate, the predominant reaction is benzoic acid + citrate<sup>3-</sup>  $\rightleftharpoons$  benzoate<sup>-</sup> + H citrate<sup>2-</sup>.

## SUMMARY

1. The solubility of benzoic acid and aspirin as a function of pH, citrate concentration, and sodium chloride concentration has been determined. In addition, the effect of sodium acetate on the solubility of benzoic acid and the effect of concentration on the dissociation constants of citric acid were investigated.

2. The interaction of sodium acetate and benzoic acid closely followed the law of mass action. The citrate interactions deviated widely from such considerations; an actual decrease in solubility was observed at high citrate concentrations. These results, however, are to be expected if the effect of ionic environment is taken into consideration.

3. Analysis of the acid-base interactions with the aid of other studies, pursued here and else-

where, suggests that complexing occurs to only a limited extent, if at all, except at very high citrate concentrations where such associations may play a predominant role in the interactions.

## REFERENCES

- (1) Bowey, A. E., *Pharm J, New Zealand*, 19, 11(1957)
- (2) Doosag, S. S., and Bhagwat, W. V., *J Indian Chem Soc*, 10, 225(1933)
- (3) Higuchi, T., Gupta, M., and Busse, L. W., *THIS JOURNAL*, 42, 157(1953)
- (4) Kolthoff, I. M., and Bosch, W., *J Phys Chem*, 36, 1685(1932)
- (5) Larsson, E., *Z physik Chem, Aht A*, 153, 496(1931)
- (6) Riesch, L., and Kulpatrik, M., *J Phys Chem*, 39, 891(1935)
- (7) Gunterberg and Schrodt, *Z physik Chem*, 135, 383(1928)
- (8) Eilila, A., *Acta Chem Scand*, 6, 1562(1952)
- (9) Kiss, A. V., and Urmancy, A., *Z physik Chem, Abt A*, 171, 257(1935)
- (10) Cohn, E. J., Heyroth, F. F., and Menkin, M. F., *J. Am Chem Soc*, 50, 696(1928)
- (11) Adell, B., *Z physik Chem, Aht A*, 187, 66(1940)
- (12) Kolthoff, I. M., and Bosch, W., *Rec trav chim*, 47, 558(1928)

## Veratrum Alkaloids XLIII\*

## The Structure of Cevadine

By S. MORRIS KUPCHAN and ADRIANO AFONSO

The structure of cevadine, the principal alkaloid of *Schoenocaulon officinale* A. Gray, has been elucidated as veracevine 3-angelate (III). Acylation of veracevine with a limited amount of 3-bromoangeloyl chloride afforded veracevine 3-(3'-bromoangelate) (II). The latter compound, on hydrogenolysis, afforded veracevine 3-angelate which was found to be identical with cevadine.

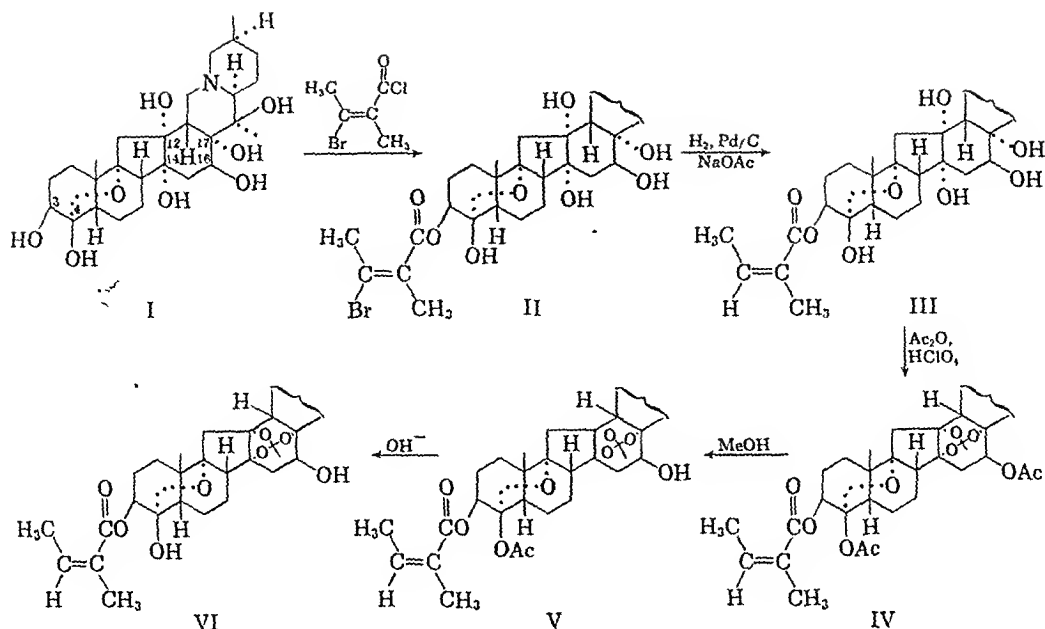
CEVADINE is the most abundant alkaloid of commercial veratrine, an insecticidal mixture of alkaloids obtained from the seeds of *Schoenocaulon officinale* A. Gray (sabadilla seed). It was first isolated by G. Merck (1) in 1855 and further characterized by Schmidt and Koppen (2) and by Wright and Luff (3). Wright and Luff hydrolyzed cevadine with dilute alcoholic alkali and isolated an amorphous alkamine and tiglic acid. Bosetti (4) and Ahrens (5) each

reported the alkaline hydrolysis of cevadine to yield an amorphous alkamine and the geometric isomer of tiglic acid, viz angelic acid. Freund and Schwarz (6) hydrolyzed cevadine with hot concentrated alcoholic potassium hydroxide, obtained the crystalline alkamine cevine and a mixture of tiglic and angelic acids, and concluded that cevadine is an angelate ester of cevine. Horst (7) disputed the latter conclusion on the basis of his finding that hydrolysis under acidic conditions gave only tiglic acid. Stoll and Seebeck (8) hydrolyzed cevadine under somewhat milder alkaline conditions than those employed previously, obtained the crystalline alkamine cevagenine and angelic acid, and concluded that cevadine is an angelate ester of cevagenine. Subsequent work by Pelletier and Jacobs (9) and by our group (10) showed that saponification of cevadine under still milder conditions afforded the alkamine veracevine (I), and supported the view that the alkamine present in cevadine is veracevine. While circumstantial evidence has made possible a tentative formulation for cea-

\* Received November 9, 1959, from the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison.

Supported in part by grants from the National Institutes of Health [H-2275 (C4)] and the Wisconsin Alumni Research Foundation.

Part XLII in the series S. M. Kupchan, C. I. Ayres, and R. H. Hensler, *J. Am. Chem. Soc.*, in press.



dine (11), the nature of the alkamine, the acid residue, and the point of attachment of the acid residue to the alkamine nucleus have not been settled unequivocally. In the present report evidence is presented for definite assignment of the veracevine 3-angelate structure (III) to cevadine.

That veracevine and angelic acid are indeed the true components of the natural ester alkaloid was established by the synthesis from veracevine of an angelate ester identical with cevadine. Veracevine (I) was treated with a limited amount of 3-bromoangeloyl chloride (12, 13, 14) to yield veracevine 3-(3'-bromoangelate) (II),  $[\alpha]_D^{21} - 4^\circ$  (ethanol). The latter compound, on hydrogenolysis, afforded veracevine 3-angelate (III), m. p. 208–209°,  $[\alpha]_D^{23} + 13^\circ$  (ethanol). This product showed no depression in melting point on admixture with an authentic sample of cevadine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

Cevadine has been regarded as a 3-acyl derivative of veracevine [see, e. g., (11)] on the basis of our observation in October 1953 (heretofore unpublished) that periodic acid cleavage affords an amorphous product which shows no  $\gamma$ -lactone absorption in the infrared. Our result indicated that the acyl residue blocked the C<sub>3</sub>, C<sub>4</sub>  $\alpha$ -ketol hemiketal system toward periodic acid attack. However, the latter experiment did not distinguish between a C<sub>3</sub> and a C<sub>4</sub> ester structure for cevadine. We deemed the presence of an acyl

moiety at C<sub>4</sub> as highly unlikely on biogenetic grounds, for no C<sub>4</sub> ester has been found among the multitude of naturally occurring ester derivatives of the veratrum alkamines zygadenine, germine, or protoverine (13–19). Nevertheless, it was considered desirable to seek experimental evidence in favor of the C<sub>3</sub> ester structure (III) by chromic acid titration experiments. Cevadine d-orthoacetate (VI) (20) demonstrated a chromic acid consumption which paralleled that of cevadine d-orthoacetate monoacetate (V) and indicated the presence of one secondary hydroxyl group. C<sub>4</sub> was thereby excluded as a site for attachment of the angelate ester of cevadine, for a C<sub>4</sub>-attachment would leave two chromic acid-sensitive secondary hydroxyl groups in cevadine d-orthoacetate. The combination of the results reported herein strongly support the veracevine 3-angelate structure (III) for cevadine.

## EXPERIMENTAL

Melting points are corrected for steam exposure. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer and chloroform was used as the solvent. Paper chromatograms were run by the descending technique employing Whatman No. 1 paper.

**Veracevine 3-(3'-Bromoangelate) (II).**—Veracevine (I, 1.02 Gm., m. p. 220–225° (decompn.) after softening at 179–183°) was dissolved in pyridine (5 cc.) and the stirred solution was treated at room temperature with 3-bromoangeloyl chloride (0.60 Gm.) (12). The mixture was stirred for sixteen hours, cooled with crushed ice, brought to pH 8 with 10% sodium carbonate solution, and extracted thoroughly

with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was dissolved in benzene and the solution was evaporated to dryness under reduced pressure; the procedure was repeated until the residue no longer smelled of pyridine. The brownish-yellow residue was triturated with ether (25 cc.) and the insoluble solid (0.36 Gm.) identified as veracevine, removed by filtration. The filtrate was evaporated to dryness and the pale yellow residue (0.74 Gm.) chromatographed on Merck acid-washed alumina (14 Gm.) The progress of the column separation was followed by paper chromatography employing the procedure of Levine and Fischbach (21) [solvent system: *n*-butyl acetate, *n*-butanol, formic acid (25:5:1 by volume)]. Elution with 50% benzene-chloroform (200 cc.) yielded an amorphous residue (0.10 Gm.) which appeared to consist of a mixture of mono and diesters. Elution with chloroform (500 cc.) yielded veracevine (3-3'-bromoangelate) (0.26 Gm.) as a pale yellow amorphous solid,  $[\alpha]_D^{25} -4^\circ$  (c 1.36 ethanol). Further elution with 2% methanol-chloroform yielded veracevine.

**Hydrogenolysis of Veracevine 3-(3'-Bromoangelate): Synthesis of Cevadine (III).**—To a suspension of 10% palladium on carbon (0.07 Gm.) in 95% ethanol (10 cc.) which had previously been saturated with hydrogen, was added a solution of veracevine 3-(3'-bromoangelate) (II, 0.14 Gm.) in ethanol (4 cc.) containing anhydrous sodium acetate (0.07 Gm.). Hydrogenation was allowed to proceed at room temperature and atmospheric pressure until one mole equivalent of hydrogen had been consumed (ca. three minutes). The catalyst was removed by filtration, washed with ethanol (10 cc.), and the filtrate evaporated to dryness under reduced pressure. The residue was suspended in ice water (2 cc.), made alkaline with 10% sodium carbonate solution, and extracted with chloroform. The chloroform extract was dried over anhydrous sodium sulfate and evaporated under reduced pressure to yield a resin (0.12 Gm.) which was crystallized from acetone-water to yield cevadine (85 mg.), m. p. 208–209°,  $[\alpha]_D^{25} +13^\circ$  (c 0.92, ethanol). The melting point was not depressed on admixture with an authentic sample of cevadine. The infrared spectra and paper chromatographic behavior of the respective samples were identical.

**Periodic Acid Oxidation of Cevadine.**—Cevadine (III, 2 Gm.), m. p. 208–210°, dissolved in methanol (25 cc.) and water (25 cc.), was treated with periodic acid (1 Gm.). After twenty hours at room temperature, the solution was concentrated under reduced pressure to remove the methanol. Ammonium hydroxide was added to pH 9 and the solution extracted repeatedly with chloroform. The chloroform solution

TABLE I.—CHROMIC ACID TITRATIONS

Alkaloid	Oxygen Equivalents Consumed, hr		
	1	2	3
Cevadine orthoacetate acetate (V)	0.83	0.93	0.93
Cevadine orthoacetate (VI)	0.85	0.95	0.95

was concentrated to about 50 cc., whereupon a colorless solid separated. The solid was filtered, identified as ammonium periodate, and rejected. The chloroform filtrate was evaporated to dryness, whereupon a colorless amorphous alkaloidal residue was obtained. The residue resisted all attempts at crystallization. The infrared spectrum of the material showed a broad (carbonyl) band in the region of 5.81–5.90  $\mu$ , but no absorption in the 5.00 to 5.80  $\mu$  region.

**Chromic Acid Titrations.**—The method described in Part XXXVIII (22) was used. Cevadine D-orthoacetate 4-acetate (V) ("monoacetyl-anhydrocevadine"), m. p. 280–281° (decompn.),  $[\alpha]_D^{25} +72^\circ$  (c 0.95, chf.), and cevadine D-orthoacetate (VI) ("anhydrocevadine"), m. p. 214–215° (decompn.),  $[\alpha]_D^{25} +97^\circ$  (c 0.98, ethanol), were prepared according to the procedures of Stoll and Seebeck (20). The results obtained on chromic acid titration of the latter compounds are summarized in Table I.

## REFERENCES

- (1) Merck, G., *Ann.*, **95**, 200 (1855).
- (2) Schmidt, E., and Koppen, R., *ibid.*, **185**, 224 (1877).
- (3) Wright, C. R., and Luff, A. P., *J. Chem. Soc.*, **32**, 338 (1878).
- (4) Bosetti, E., *Arch. Pharm.*, **221**, 82 (1883).
- (5) Ahrens, F. B., *Ber.*, **23**, 2700 (1890).
- (6) Freund, M., and Schwarz, H., *ibid.*, **32**, 800 (1899).
- (7) Horst, P., *Chem. Ztg.*, **26**, 334 (1902).
- (8) Stoll, A., and Seebeck, E., *Helv. Chim. Acta*, **35**, 1270 (1952).
- (9) Pelletier, S. W., and Jacobs, W. A., *J. Am. Chem. Soc.*, **75**, 3248 (1953).
- (10) Kupchan, S. M., Lavie, D., Deliwala, C. V., and Andoh, B. Y. A., *ibid.*, **75**, 5519 (1953).
- (11) Fieser, L. E., and Fieser, M., "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, p. 888.
- (12) Kupchan, S. M., and Afonso, A., *J. Org. Chem.*, in press.
- (13) Kupchan, S. M., and Afonso, A., *THIS JOURNAL*, **48**, 731 (1959).
- (14) Kupchan, S. M., and Ayres, C. I., *ibid.*, **48**, 735 (1959).
- (15) Kupchan, S. M., *J. Am. Chem. Soc.*, **81**, 1921 (1959).
- (16) Kupchan, S. M., *ibid.*, **81**, 1925 (1959).
- (17) Kupchan, S. M., and Ayres, C. I., *THIS JOURNAL*, **48**, 440 (1959).
- (18) Kupchan, S. M., and Ayres, C. I., *J. Am. Chem. Soc.*, in press.
- (19) Kupchan, S. M., Ayres, C. I., and Hensler, R. H., *ibid.*, in press.
- (20) Stoll, A., and Seebeck, E., *Helv. Chim. Acta*, **35**, 1942 (1952).
- (21) Levine, J., and Fischbach, H., *THIS JOURNAL*, **44**, 513 (1955).
- (22) Kupchan, S. M., Ayres, C. I., Neeman, M., Hensler, R. H., Masamune, T., and Rajagopalan, S., *J. Am. Chem. Soc.*, in press.

# Some Pharmaceutical Properties of Novobiocin\*

By JOHN D. MULLINS and THOMAS J. MACEK

Novobiocin (Cathomycin) is an antibiotic produced in nutrient media by *Streptomyces spheroides*. Physical characteristics of the amorphous acid form of novobiocin were studied. The amorphous acid is biologically active, but proved metastable in aqueous suspension, reverting on standing to a less soluble, inactive crystalline form. Several distinct steps were identified during the process of reversion; the rate of reversion was found to be temperature dependent. Methods for suppressing crystallization of the amorphous acid are discussed. The properties of amorphous calcium novobiocin in formulation of aqueous suspensions were studied.

NOVOBIOCIN is an antibiotic substance produced in nutrient media by *Streptomyces spheroides*. Several authors have described its chemical, microbiological, and pharmacologic properties (1-7).

The antibiotic normally is produced as the crystalline monosodium salt. This form is absorbed following oral or parenteral administration and is effective against strains of *Streptococcus*, *Staphylococcus*, *Proteus*, *Diplococcus*, and *Pasturella*. *Staphylococci* seem to be particularly sensitive, as are several strains of penicillin-resistant *Streptococci*.

Novobiocin usually occurs as a white to cream-colored amorphous solid or a pale yellow crystalline solid, melting at 153 to 158°, with decomposition. The antibiotic is a weak dibasic acid having pK values of 3.8 and 9.4. It is soluble in methanol, ethanol, butanol, acetic acid, and dioxane; it is insoluble, or only slightly soluble, in ether, benzene, carbon tetrachloride, and chloroform. Although novobiocin acid is insoluble in water, both the mono and disodium salts are very soluble, but the rate of solution is quite slow.

Solutions of monosodium novobiocin are slightly alkaline and are incompatible with acids and acid salts. The antibiotic forms essentially insoluble salts with heavy metal ions. Insoluble salts are also formed by reaction with several amines such as procaine, phenylethylamine, morpholine, and N,N'-dibenzylethylenediamine. Novobiocin also forms insoluble salts with basic antibiotics such as streptomycin, neomycin, and kanamycin. Several of these have been prepared and characterized (8).

In 0.1 N sodium hydroxide solution novobiocin exhibits an absorbance maximum at 307

m $\mu$  with  $E(1\%, 1 \text{ cm.}) = 600$ . In phosphate buffer at pH 7.0 the absorbance maximum occurs at 305 m $\mu$  with  $E(1\%, 1 \text{ cm.}) = 343$ . In acidic methanol (0.1 N hydrochloric acid in 90% methanol) the absorbance maximum occurs at 305 m $\mu$  with  $E(1\%, 1 \text{ cm.}) = 384$ .

Novobiocin is optically active showing the following rotational characteristics:

$$[\alpha]_D^{25} = -44^\circ \quad (C = 1\%, \text{ pyridine})$$

$$[\alpha]_D^{25} = -29^\circ \quad (C = 1\%, 1 \text{ N sodium hydroxide})$$

Titration curves for monosodium novobiocin with citric acid and with hydrochloric acid are shown in Fig. 1.

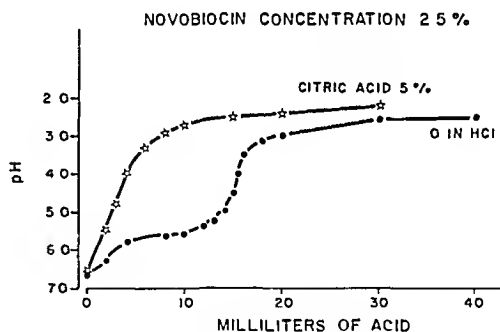


Fig. 1.—Novobiocin titration.

## EXPERIMENTAL

**Crystalline and Amorphous Novobiocin.**—When an aqueous solution of the sodium salt is acidified novobiocin acid is usually obtained as an amorphous precipitate. However, the amorphous solid is metastable and is readily converted to a more stable crystalline form. The rate of conversion is temperature dependent and proceeds quite rapidly at higher temperatures.

Apart from the more obvious physical differences between crystalline and amorphous forms, an important though unexpected difference has been observed in biological availability. The crystalline acid is poorly absorbed and does not produce therapeutically adequate systemic levels of novobiocin following oral administration. The amorphous acid, on the other hand, is readily absorbed and is ther-

\* Received August 21, 1959, from Merck Sharp and Dohme Research Laboratories, West Point, Pa.

The authors gratefully acknowledge the cooperation of Dr. John Baer and Mrs. Helen Skeggs in performing the biological tests, Dr. D. E. Williams for the X-ray measurements, and Mr. R. McGaughran for the infrared measurements. All these individuals are members of the staff of the Merck Sharp and Dohme Research Laboratories.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.



apeutically active. This difference in availability was thought to be related to differences in solubility in water and aqueous systems. Experiments to test the hypothesis were performed by shaking an excess of crystalline or amorphous novobiocin acid, both essentially less than  $10\ \mu$  in particle size, with 400 ml. of 0.1 *N* hydrochloric acid at 25°. At hourly intervals samples were taken; ultraviolet absorbance was measured after filtration. When it became available, the same experiment was performed with amorphous calcium novobiocin. This salt was later found to be biologically active although not particularly soluble.

The data in Fig. 2, corrected for volume changes, indicate that both amorphous novobiocin acid and the amorphous calcium salt exhibited greater absorbance as a function of time than did the crystalline solid. This was interpreted to mean that the amorphous solids were at least tenfold more soluble in 0.1 *N* hydrochloric acid than was the crystalline solid. This difference in solubility might be expected to favor the absorption of the amorphous solid from the gastrointestinal tract.

Data showing differences in novobiocin plasma levels in dogs following oral administration of 12.5 mg./Kg. of each of amorphous novobiocin, amorphous calcium novobiocin, and crystalline novobiocin are shown in Table I.

Blood levels obtained with the sodium salt as a control, are also shown. Satisfactory absorption of the monosodium salt suggests that the free acid formed when this salt enters the stomach is either

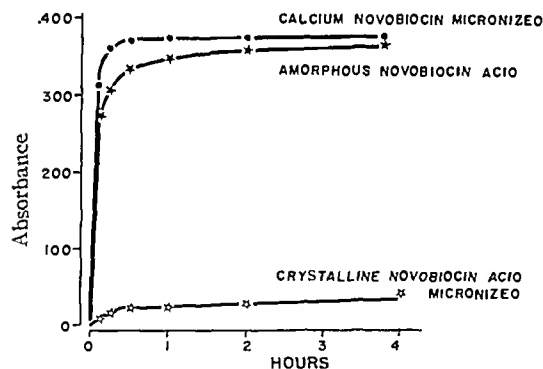


Fig. 2.—Absorbance of novobiocin in 0.1 *N* HCl at 305  $m\mu$ .

TABLE I.—NOVOBIOCIN PLASMA LEVELS IN DOGS FOLLOWING ORAL ADMINISTRATION OF DIFFERENT SOLID FORMS<sup>a</sup>

Hours after Dose	Sodium Novobiocin, mcg./ml. Plasma	Amorphous Novobiocin (Acid), mcg./ml. Plasma	Calcium Novobiocin, mcg./ml. Plasma	Crystalline Novobiocin (Acid)
1/2	0.5	5.0	9.0	b
1	0.5	40.6	16.4	b
2	14.6	29.5	26.8	b
3	22.2	22.3	19.0	b
4	16.9	23.7	15.7	b
5	10.4	20.2	13.8	b
6	6.4	17.5	10.0	b

<sup>a</sup> Dose = 12.5 mg./Kg.

<sup>b</sup> Not detectable

amorphous or of some other physical form suitable for absorption.

**Crystallization of Suspensions.**—Instability of amorphous novobiocin can be illustrated best by a description of the behavior of an aqueous suspension. When freshly prepared by acidification of a solution of the sodium salt, a suspension of amorphous novobiocin is white in color, the solid is gel-like and well suspended. The particles are approximately 1 to 5  $\mu$  in size. On standing, however, the suspension gradually becomes more dense, particle size increases, and there is greater settling of the suspended phase.

Microscopic examination of the suspension during the course of these changes reveals several other intermediate steps in the transformation process. A change in the amorphous particles is first revealed by the appearance of spheroid particles. These appear to be formed by the coalescence of a number of minute amorphous particles which become fixed together. The clustered particles tend to become more dense and show some increase in color; they assume a roughly spherical shape, and continue to increase in size in a roughly symmetrical manner. In larger spheroids concentric growth rings sometimes become visible. The spheroid particles attain a maximum diameter of approximately 30  $\mu$ . At this point by gross examination, the suspension is definitely more yellow in color and sedimentation rate has increased.

The second or crystallization step first becomes visible microscopically by the appearance of needle or rod-like crystals vertical to the periphery of the spheroids. Crystal growth then proceeds inward toward the center of the spheroid. Fragility of the spheroids increases with depth of crystallization until soon the novobiocin predominantly is present in suspension as intact or fragmented rosettes of needle-like crystals. The photomicrographs in Fig. 3 illustrate the various steps in the crystallization process.

The process of crystallization of novobiocin also has been examined by X-ray diffraction studies using a Norelco X-ray diffractometer. Figure 4 illustrates the intensity of X-ray diffraction from a crystalline sample as contrasted with little or no diffraction by a sample of amorphous novobiocin. It is interesting to note that spheroids are no longer amorphous by X-ray diffraction nor are they fully crystalline.

Examination of the infrared spectra of both the crystalline and amorphous forms of novobiocin revealed only minor differences and the spectra for the two forms were practically indistinguishable. The spectrum for the crystalline solid showed some evidence of increase in bond strength at several points.

The process of crystallization in suspensions was observed to be irreversible, as expected. On the other hand, rate of crystallization appeared to vary considerably with temperature. Indeed at room temperature crystallization could not be induced by vigorous shaking and scratching even with the addition of crystalline seed. Experiments, therefore, were conducted to determine the length of time required for spontaneous crystallization to take place in aqueous suspensions containing a therapeutically-useful concentration of amorphous novobiocin.

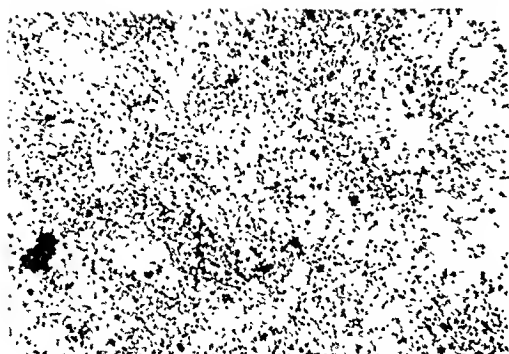
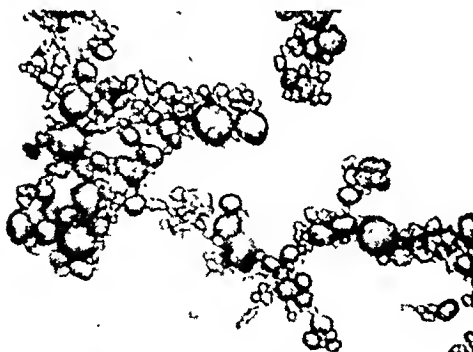
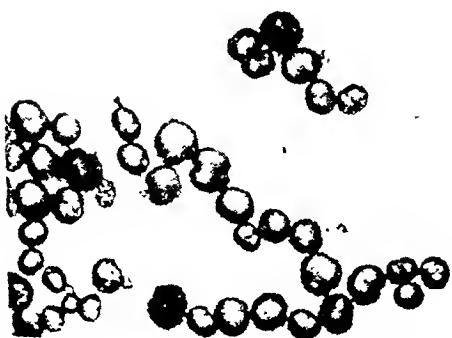
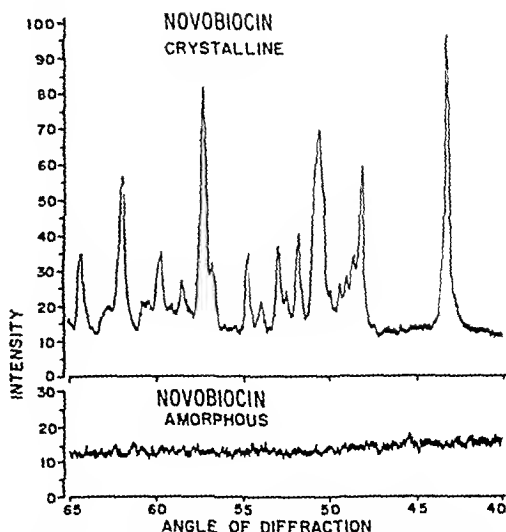
Fig. 3A.—Amorphous novobiocin ( $\times 344$ ).Fig. 3B.—Beginning spheroid formation ( $\times 344$ ).Fig. 3C.—Spheroid formation ( $\times 344$ ).Fig. 3D.—Spheroids with peripheral crystallization ( $\times 344$ ).

Fig. 4.—X-ray diffraction; amorphous and crystalline novobiocin.

A solution of sodium novobiocin, equivalent to 2.5% novobiocin acid, was adjusted to pH 3.5 with hydrochloric acid. Samples of the suspension of amorphous novobiocin acid which was formed were subdivided into glass bottles for storage at several temperatures. The specimens of stored suspensions were examined microscopically for crystallization at frequent intervals during storage. The end point was taken as the presence of both spheroids and crystals on the same microscopic field. Crystallization was observed to occur within a few hours at 60°, but only after six months at 25°. The data are shown in Table II.

TABLE II.—APPROXIMATE TIME REQUIRED FOR CRYSTALLIZATION OF NOVOBIOCIN SUSPENSION

Storage Temperature, °C	Crystallization Time
60	4-6 hours
50	24 hours
37	22 days
25	6 months

As a continuation of these studies it was observed that suspensions containing 1.25% amorphous novobiocin required a longer time for crystallization. At a concentration of 0.625% amorphous novobiocin the time required for crystallization was approximately twice that found for the 2.5% samples.

**Stabilization of Suspensions.**—Even though aqueous suspensions containing 2.5% or more of amorphous novobiocin were found physically stable for six months at room temperature, these represented thermodynamically unstable systems. Crystallization of such suspensions in a shorter period of time or under more adverse conditions of storage and shipment could not be dismissed. On the other hand, crystalline acid was inactive and sodium novobiocin, although useful orally, could not be dispensed in ready-made liquid form because of chemical instability.

Inasmuch as the crystallization of suspensions of amorphous novobiocin was observed to be a step-wise process, it seemed reasonable that crystallization could be controlled further with additives. Such procedures certainly are well-known in the control of many crystallization operations.

Accordingly, a search was conducted for additives that would significantly retard or even prevent crystallization of aqueous suspensions of amorphous novobiocin. Suspensions of the amorphous acid at 2.5% concentration were prepared by adjusting aqueous solutions of sodium novobiocin to pH 3.5 with hydrochloric acid. Additives to retard crystal formation were generally present during acidification. In a few instances they had to be added in aqueous solution immediately afterward. The temperature and rate of addition of hydrochloric acid, rate of stirring, and volume of sample were kept uniform. All samples, together with appropriate controls containing no additives, were stored in glass bottles at 50°. These were examined microscopically at frequent intervals for signs of crystallization or spheroid formation. A list of additives tested and their effect on crystallization time is given in Table III.

TABLE III—EFFECT OF ADDITIVES ON RATE OF CRYSTALLIZATION OF AMORPHOUS NOVOBIOCIN ACID IN AQUEOUS SUSPENSION

Additive	Rate of Crystallization
Benzalkonium chloride	Increased
Tween series <sup>a</sup>	Increased
Pluronic F-68 <sup>b</sup>	Increased
Polyethylene glycol esters	Increased
Sucrose esters	Increased
Polyethylene glycol series	Increased
Lecithin	Increased
Dextran	Decreased slightly
Gelatin	Decreased slightly
Carboxymethylcellulose	Decreased slightly
Starch	Decreased slightly
Hydroxyethylcellulose <sup>c</sup>	Decreased slightly
Cyanoethylated starch	Decreased slightly
Acacia	Decreased slightly
Tragacanth	Decreased slightly
Casein	Decreased slightly
Lactalbumen	Decreased slightly
Bentonite	Decreased slightly
Methylcellulose	Decreased markedly
Polyvinylpyrrolidone	Decreased markedly
Cellulose acetate hydrogen phthalate	Decreased markedly
Sodium alginate	Decreased markedly
Propylene glycol algin <sup>d</sup>	Decreased markedly

<sup>a</sup> Atlas Powder Co.

<sup>b</sup> Wyandotte Chemicals Corp.

<sup>c</sup> Cellosize Carbide and Carbon Chemical Co.

<sup>d</sup> Kelcolloid Kelco Co.

The data show the surface active agents as a class, regardless of ionic character, generally increased the rate at which crystallization took place.

A number of water-soluble or water-dispersible gums and substances generally regarded as protective colloids were found ineffective in retarding this crystallization process. Only few agents provided adequate protection against crystallization for really significant periods of time. Further, time of

crystallization was found dependent upon concentration of the additive, as is shown in Table IV with

TABLE IV—EFFECT OF METHYLCELLULOSE CONCENTRATION ON NOVOBIOCIN CRYSTALLIZATION RATE

Concentration of Methylcellulose 1:500 c p s as % of Total Volume	Time Required for Crystallization days 50°	Time Required for Crystallization days 37°
0	1	22
0.1	2	25
0.25	3	35
0.50	5	60
0.75	7	About 90
1.00	25	>365

various concentrations of methylcellulose. The best agents for stabilizing suspensions of amorphous novobiocin against crystallization were methylcellulose, several types of alginic acid derivatives, such as sodium alginate or propylene glycol algin and polyvinylpyrrolidone.

**Suspension of Calcium Novobiocin.**—The preparation of the calcium salt of novobiocin solved numerous problems previously encountered with the acid.

Calcium novobiocin proved to be a tasteless salt that was fully biologically active and perfectly stable in aqueous suspension though amorphous. Data showing solubility in dilute acid and availability in dogs were given previously.

Even though crystalline calcium novobiocin has been obtained from organic solvents, the amorphous salt is obtained in water by precipitation of sodium novobiocin with calcium chloride. Aqueous suspensions of the amorphous calcium salt have failed to crystallize in our laboratory studies even after twenty-four months of storage at room temperature and at 37°. On this basis, the addition of substances to retard crystallization has not been found necessary.

## SUMMARY

1 The pharmaceutical properties together with some of the physical and chemical properties of novobiocin have been described.

2 The characteristics of the metastable amorphous form of novobiocin have been discussed. Different additives for stabilizing this biologically-active, metastable form are described.

## REFERENCES

- (1) Kaczka, E. A., Wolf, F. J., Rathe, F. P., and Folkers, K. *J. Am. Chem. Soc.* **77**, 6401 (1955).
- (2) Hoeksema, H., Johnson, J. L., and Hinman, J. W. *ibid.* **77**, 6710 (1955).
- (3) Wallick, H., Harris, D. A., Reagan, M. A., Ruger, M., and Woodruff, H. B., "Antibiotics Annual 1955-56," Medical Encyclopedia Inc., New York, N. Y. 1956, p. 909.
- (4) Verwey, W. F., Miller, A. K., and West, M. K. *ibid.* p. 924.
- (5) Smith, C. G., Dietz, A., Sokolski, W. T., and Savage, G. M., *Antibiotics & Chemotherapy* **6**, 135 (1956).
- (6) Wilkins, J. R., Lewis, C., and Barbiers, A. R. *ibid.* **6**, 149 (1956).
- (7) Taylor, R. M., Miller, W. L., and VanderBrook, M. J., *ibid.* **6**, 162 (1956).
- (8) Chaiet, L., and Wolf, F. *ibid.* **7**, 231 (1957).

# The Effect of Thixotropy on Suspension Stability\*

By ERNEST C. FOERNZLER, ALFRED N. MARTIN, and GILBERT S. BANKER

This study was undertaken to investigate the effect of thixotropy on physical suspension stability. Two thixotropic suspending agents, bentonite and attapulgite, were evaluated in systems of varying concentrations for thixotropy, plastic viscosity, and yield value by employing a modified Stormer viscometer. A technique involving centrifugation was developed for the evaluation of stability. In the case of both suspending agents employed, the correlation of physical suspension stability and the degree of thixotropy of the suspending agent indicated that sedimentation velocity was directly proportional to the reciprocal of thixotropic area.

**D**UE TO THE NATURE of pharmaceutical suspensions and the fact that they are to be used as dosage forms, physical stability is of utmost importance. In attempting to make calculations concerning the physical stability of a pharmaceutical suspension, however, it is usually found that an extremely complex system must be dealt with, the parameters of which are in many cases quite difficult to measure and often impossible to express mathematically. Furthermore, the rates of involved physical processes are equally difficult to evaluate (1). Here it is earnestly felt that time and effort may be conserved if the experimentation is directed toward rheological methods.

The colloidal phenomenon of thixotropy is an important physical property of a variety of pharmaceutically acceptable suspending agents. Through utilization of thixotropy in the preparation of liquid suspensions it is possible to minimize or eliminate sedimentation and coagulation of the insoluble constituents which the product contains. Thus, a knowledge of the flow properties of thixotropic suspending agents should provide an insight into the formulation and development of suspended products of superior physical stability. Many pharmaceutical suspending agents are known to possess thixotropic flow properties. Among these are bentonite (2), hectorite (3), Veegum<sup>1</sup> (4), attapulgite (5), sodium carboxymethylcellulose (6), and Carbopol 934<sup>2</sup> (7). It is unfortunate that there exists at the present time no generally accepted method or specific conditions under which rheological analyses for thixotropic flow should be made. However, numerous publications have appeared in which the investigators have taken advantage of rheological evaluation procedures for analysis of

thixotropic pharmaceutical systems (2, 6, 8-10). Although there will undoubtedly be some disagreement among workers representing methods of analysis which are not standardized, the information obtained from proper rheological evaluation of systems of this type is very useful for providing data by which the systems may be analyzed and controlled.

This study (a) evaluates the flow properties of two thixotropic suspending agents suitable for use in pharmaceutical systems, (b) evaluates the physical stability of medicinal products prepared with these suspending agents, and (c) attempts to obtain a mathematical relationship which would correlate thixotropy and physical suspension stability.

The two suspending agents employed in this project were bentonite, U. S. P.,<sup>3</sup> and Pharmasorb Colloidal<sup>4</sup> (activated attapulgite).

## EXPERIMENTAL

**Preparation of Suspension Bases.**—Before preparing the suspension bases, both suspending agents were analyzed for moisture content by a standard Karl Fischer titrimetric method with an electrometric end point determination. Analysis of three samples for each suspending agent indicated moisture contents of 6.59 and 16.2%, respectively, for bentonite and Pharmasorb. Following moisture correction, bentonite suspension bases were prepared in concentrations of 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, and 7.0% (w/v) by adding the appropriate quantity of clay slowly in divided portions to 800-900 ml. of hot distilled water without stirring (U. S. P. method). The product was then allowed to hydrate at room temperature (23 to 27°) for twenty-four hours. At the end of the twenty-four hour hydration period the product was mixed at 3,600  $\pm$  400 r. p. m. with a Waring Blendor<sup>5</sup> of 1 L. capacity for ten minutes. The volume of the product was then increased to 1,000 ml. and the contents mixed at the previous speed for an additional five minutes.

In the case of Pharmasorb Colloidal, the manufacturer recommends that the most stable suspension

\* Received August 21, 1959, from the School of Pharmacy, Purdue University, West Lafayette, Ind.

Abstracted from a thesis submitted to the faculty of Purdue University in partial fulfillment of the requirements for the degree of Master of Science.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

<sup>1</sup> R. T. Vanderbilt Co., New York, N. Y.

<sup>2</sup> H. F. Goodrich Co., Cleveland, Ohio.

<sup>3</sup> Fisher Scientific Co., Pittsburgh, Pa.

<sup>4</sup> Minerals and Chemicals Corp. of America, Menlo Park, N. J.

<sup>5</sup> Waring Products Corp., New York, N. Y.

bases be prepared by presoaking and/or pregelling the clay in water at 25° for a period of twelve to eighteen hours followed by stirring the mixture and diluting the pregelled concentrate (5). The Pharmasorb bases were therefore prepared at concentrations of 5.0, 6.0, 7.0, 8.0, and 9.0% (w/v) by adding to the appropriate quantity of clay a sufficient quantity of distilled water (25°) so that it just completely moistened the clay. The moistened clay was then allowed to soak for twenty-four hours at room temperature. Upon completion of the presoaking period, 800 ml. of distilled water was added, the product was mixed at 3,600  $\pm$  400 r. p. m. for ten minutes with the Waring Blendor, the volume was increased to 1,000 ml., and the contents mixed for an additional five minutes as for the bentonite bases.

After preparation, all suspension bases were stored at room temperature in tightly closed, amber glass containers and allowed to hydrate further and age for at least fourteen days before rheological or stability measurements were begun. No preservative was employed in the bases since those acceptable for use were found to produce foaming during preparation of the bases, thereby creating undesirable conditions for rheological evaluation. The absence of a preservative did not affect the results over the period of experimentation. Although the pH of the bases was determined after preparation and at thirty-day intervals during the time the bases were employed, pH itself was not considered a variable in this study.

**Rheological Evaluation.**—A Stormer viscometer, properly modified for study of non-Newtonian flow, was employed for rheological evaluation in this project. Fischer has adequately described the modifications necessary in this respect (11). All rheological measurements were made at 25.0  $\pm$  0.1° through application of a calibrated thermometer and a constant temperature bath. Before use, the viscometer was adequately calibrated with Newtonian standardization oils for use in the range of viscosity below one poise. Calculations used to obtain the instrumental constants  $K$ ,  $K_r$ ,  $K_f$ , and  $K_v$  and the working equations necessary for computing plastic viscosity ( $\bar{U}$ ) and yield value ( $f$ ) were identical to those of Fischer (11). The instrumental constant  $K_v$  was evaluated for each flow curve from the appropriate weight/r. p. m. ratio for correction of the end-effect and consideration of the effective bob length (12).

After preliminary investigation of several procedures for quantitative determination of thixotropy, it was decided that the procedure most applicable to the manually operated Stormer viscometer was the production of a single hysteresis loop with accurate control of (a) the maximum rate of shear and (b) the time intervals between successive changes of weights. The maximum rate of shear was chosen at 400 r. p. m. (381.2 sec.<sup>-1</sup>), while the time intervals between points on the curves were constant sixty-second periods. Flow curves were accepted for comparison only if a top rate of shear of 400  $\pm$  5 r. p. m. was obtained. Although the time for production of the upcurve was not held exactly constant for each flow curve, it was felt that since all curves were made in exactly the same manner, this factor was at least approximately constant.

History effects (i. e., past treatments of the

samples) were satisfactorily eliminated by allowing each sample to remain undisturbed in the viscometer cup and bob for two hours prior to beginning the flow curves. During this time it was necessary to prevent evaporation of moisture from the sample. This was accomplished by construction of a plastic, rubber lined top for the cup and bob. Following the experimental procedure, a plot of rate of shear *versus* shearing stress was made for each sample, and the parameters of plastic viscosity and yield value were calculated. The area of the hysteresis loop was then measured directly in square inches by application of a Dietzgen-Ott compensating planimeter,<sup>6</sup> and the resulting value was designated as the thixotropic area. Samples for each concentration of the suspending agents were run from four to ten times at two-to three-day intervals over a period of fourteen to twenty-one days, and mode values were taken to obtain the average rheological parameters.

**Evaluation of Stability.**—On standing, an initially uniform and relatively dense suspension of fine particles begins to settle at a constant rate with a well defined interface between the supernatant clear liquid and the settling particles. The rate at which the height of the interface changes with respect to time is known as the sedimentation velocity of the system. Preliminary work indicated that stability studies on the shelf at normal gravitational force would require periods ranging from six months to one year and more for evaluation of sedimentation velocities. Therefore, it was decided that accelerated studies should be employed. Multiplication of gravitational force was accomplished through application of a size 1, type SB International centrifuge.<sup>7</sup> The instrument was equipped with a hand brake, a rheostat speed control, a number 240 eight-place head, and a Waltham tachometer. Fifty-milliliter, Pyrex-glass graduated centrifuge tubes with short conical bottoms were used to contain the samples which were carried through radial distances of 10.0 and 20.1 cm. as measured from the shaft to the free surface and the tip of the sample respectively. Samples were prepared for centrifugation by incorporating 10% (w/v) zinc oxide (powder), U. S. P., in the suspension bases of varying concentrations of suspending agents. Incorporation was accomplished by sixty seconds of levigation with a small portion of the base and ten minutes of mixing with the remainder of the base by a laboratory power mixer. During centrifugation the centrifuge tubes were covered with polystyrene plastic sheeting to minimize evaporation and prevent aging effects. Each run in the centrifuge was made with four samples which were adequately balanced for minimum vibration, and care was taken when stopping the instrument to avoid convection currents.

Samples prepared with the bentonite bases were evaluated at 500, 750, 1,000 and 1,500 r. p. m., while those prepared with Pharmasorb were studied at 250, 500, 750, and 1,000 r. p. m. Following centrifugal sedimentation of the samples, graphs were constructed for evaluation of the initial free-fall sedimentation velocities by plotting the sediment volume *versus* time. In some cases it was

<sup>6</sup> E. Dietzgen Co., New York, N. Y.

<sup>7</sup> International Equipment Co., Boston, Mass.

necessary to employ the method of least squares for evaluation of the slopes of the sedimentation curves.

## RESULTS AND DISCUSSION

Figures 1 and 2 show typical examples of the flow curves obtained in this study. Table I lists the average rheological values obtained for each suspension base and also indicates the sedimentation velocities obtained at 500 r. p. m. (a centrifugal force of approximately  $1.85 \times 10^6$  dynes) after incorporation of 10% zinc oxide. The sedimentation rates obtained at 500 r. p. m. were used for correlation of stability and thixotropy since these values appeared to be the most consistent and since they were obtained for all but two (bentonite 6 and 7%) of the suspension bases evaluated. An attempt was made to extrapolate the sedimentation velocities to normal gravitational force by the use of semilog plots, but it was found that a sufficient number of points could not be obtained for valid extrapolation with the equipment employed.

By plotting the sedimentation velocities against the respective thixotropic areas it was found that hyperbolic functions appeared to exist for both suspending agents. Further investigation through plotting the sedimentation velocities *versus* the reciprocal of thixotropic area, Figs 3 and 4, appeared to verify the hyperbolic relationships. These functions may be expressed in the form

$$V \cdot T = K \quad (\text{Eq. 1})$$

where  $V$  is the sedimentation velocity,  $T$  is the thixotropic area, and  $K$  is a material constant. Evaluation of the constant  $K$  resulted in values of 0.027 and 0.048 for bentonite and Pharmasorb, re-

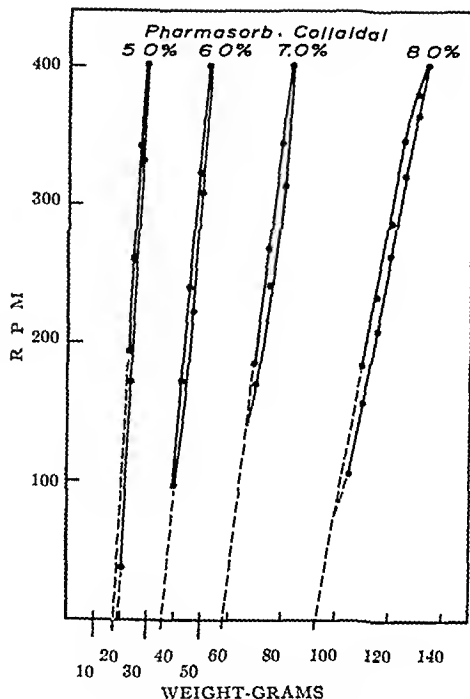


Fig. 2—Typical Pharmasorb Colloidal flow curves. (Downcurves on the left.)

TABLE I—AVERAGE RHEOLOGICAL PARAMETERS AND SEDIMENTATION VELOCITIES AT 500 R P M IN ML/MIN.

Concn., % w/v	Thixotropic Area, Sq. In.	$U$ Centipoise	$f$ Dynes/cm <sup>2</sup>	Sedimentation Velocity
Bentonite				
2.5	0.84	6.47	1.61	0.061
3.0	1.09	7.59	3.24	0.025
3.5	2.02	11.71	7.60	0.018
4.0	2.49	13.60	10.40	0.010
4.5	3.67	18.80	28.70	0.007
5.0	4.51	24.55	33.39	0.003
6.0	8.26	37.79	80.12	
7.0	13.18	59.64	174.67	
Pharmasorb Colloidal				
5.0	0.06	4.23	32.15	0.800
6.0	0.14	6.37	59.79	0.400
7.0	0.24	10.76	101.79	0.200
8.0	0.49	16.92	175.64	0.100
9.0	0.84	22.67	245.25	0.050

spectively. Thus it can be said that as a first approximation the sedimentation velocity is directly proportional to the reciprocal of the thixotropic area. It is felt that the source of variation from true linearity in the case of both bentonite and Pharmasorb was due largely to inaccurate determination of the sedimentation velocities.

The relationship obtained from Figs 3 and 4 is of both theoretical and practical interest. Assuming that the sedimentation velocity of a particular suspension base containing some therapeutic ingredient could be evaluated under accelerated condi-

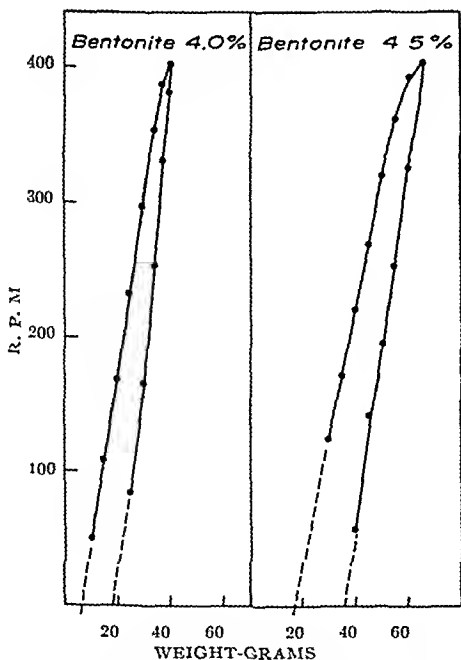


Fig. 1.—Typical bentonite flow curves (Downcurves on the left.)

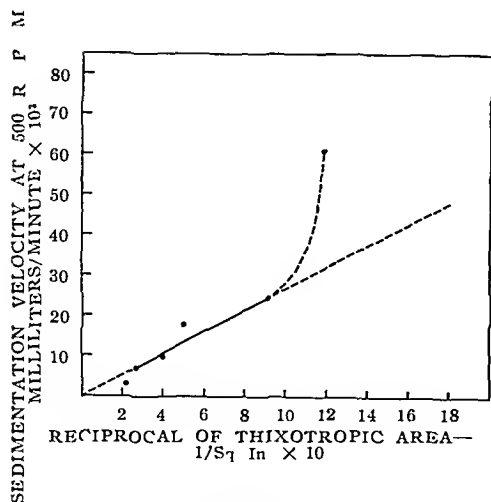


Fig. 3.—The stability-thixotropy relationship for bentonite

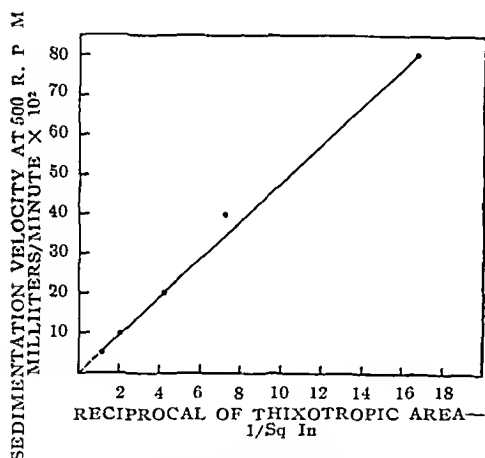


Fig. 4.—The stability-thixotropy relationship for Pharmasorb Colloidal

tions and that the rate of sedimentation could satisfactorily be extrapolated to normal conditions, the degree of thixotropy required for optimum physical stability could be obtained. Conversely, it might be possible to predict the physical stability of a given suspension base after a rheological evaluation of thixotropy had been made. Of course, other properties such as plastic viscosity and yield value and the concentration of the system must also be taken into consideration before a more general correspondence between physical stability and rheological properties can be expected. Investigation of the effect of plastic viscosity and

yield value on sedimentation velocity and of concentration on rheological properties is in progress, and an attempt to relate physical suspension stability mathematically to all three rheological properties has been planned for future discussion.

## SUMMARY AND CONCLUSIONS

An attempt has been made to correlate thixotropy and physical suspension stability for two thixotropic pharmaceutical suspending agents, bentonite and Pharmasorb Colloidal.

A properly modified Stormer viscometer was used for rheological evaluation, and a technique involving centrifugation was employed for evaluation of stability under accelerated conditions.

An attempt to extrapolate sedimentation velocities obtained at accelerated rates to normal gravitational force did not appear to provide consistent results.

In the case of both suspending agents employed the correlation of physical suspension stability and the degree of thixotropy of the suspending agent indicated that the sedimentation velocity was directly proportional to the reciprocal of the thixotropic area, or that physical stability is directly proportional to thixotropy.

As a result of this work it may be concluded that both suspension evaluation and suspension formulation may be benefited by the application of proper rheological procedures.

Further investigation of physical suspension stability as a function of thixotropy, plastic viscosity, yield value, and concentration is currently in progress.

## REFERENCES

- (1) Higuchi, T, *THIS JOURNAL*, 47, 657 (1953)
- (2) Bernstein, H. B., and Barr, M., *ibid.*, 44, 375 (1953)
- (3) Macaloid Brochure, The Inertco Co., San Francisco, Calif., p. A 2
- (4) Veegum Technical Bulletin, No 122, R. T. Vanderbilt Co., New York, N. Y.
- (5) Pharmasorb Technical Bulletin, No MDX 101, Minerals and Chemical Corp. of America, Menlo Park, N. J., 1958
- (6) deButts, E. H., Hudy, J. A., and Elliott, J. H., *Ind Eng Chem*, 49, 94 (1957)
- (7) Dittmar, C. A., *Drug and Cosmetic Ind.*, 81, 446 (1957)
- (8) Kostenbauder, H. B., and Martin, A. N., *THIS JOURNAL*, 43, 401 (1954)
- (9) Bernstein, H. B., and Barr, M., *ibid.*, 46, 41 (1957)
- (10) Ober, S. S., Vincent, H. C., Simon, D. E., and Fredrick, K. J., *ibid.*, 47, 667 (1958)
- (11) Fischer, E. K., "Colloidal Dispersions," John Wiley & Sons, New York, N. Y., 1950, pp 147-180
- (12) Hamlow, E. E., "The Correlation of Several Rheological Methods for Measuring Newtonian and Non Newtonian Materials," Ph D Thesis, Purdue University, West Lafayette, Ind., 1958

## Notes

### A Note on the Photolytic Degradation of Anti-Inflammatory Steroids\*

By W. E. HAMLIN, T. CHULSKI, R. H. JOHNSON, and J. G. WAGNER

PREVIOUSLY DESCRIBED (1-3) photolytic degradations of the A-ring of  $\Delta^4$ -3-keto steroids and  $\Delta^{1,4}$ -3-keto steroids are of interest pharmaceutically since: (a) alcoholic solutions of such steroids are used in assay procedures and the steroid might degrade therein, and (b) ultraviolet light might deleteriously affect steroid-containing dosage forms.

Savard, *et al* (1) showed that exposure of  $\Delta^4$ -3-keto steroids to ultraviolet light caused saturation of the 4,5-double bond, or migration of the double bond, or both. Barton and Taylor (2) demonstrated that exposure of the  $\Delta^{1,4}$ -3-keto steroid, prednisone acetate, caused a complicated rearrangement in the A-ring of the steroid.

We have studied the effects of exposure of alcoholic solutions of hydrocortisone, prednisolone, and methylprednisolone to ordinary fluorescent laboratory lighting and of certain ophthalmic preparations, which contain steroid alcohols solubilized in aqueous solutions of Triton WR 1339,<sup>1</sup> to various sources of ultraviolet light. The commercial ophthalmic preparations studied were eye drops Optef 0.2%<sup>2</sup> which contains 0.2% hydrocortisone (Cortef<sup>2</sup>) and eye drops Neo-Deltec, 0.2%<sup>2</sup> which contains 0.2% prednisolone (Delta-Cortef<sup>2</sup>) and 0.5% neomycin sulfate. These sterile solutions are described in the patent of Johnson (4). The effects of exposure of these solutions, stored in different types of containers, to various sources of ultraviolet light were studied.

#### EXPERIMENTAL

**Assay Methods.**—The ultraviolet spectrophotometric method (5) and the isonicotinic acid hydrazide method (6) are specific for the  $\Delta^4$ -3-keto and  $\Delta^{1,4}$ -3-keto groupings in the A-ring of the steroids. A significant decrease in potency as determined by these assay methods indicates degradation has occurred in the A-ring of the steroid. The 2,3,5-triphenyl tetrazolium chloride (tetrazolium) assay (7) involves reaction of the ketol side chain of the steroids. A significant decrease in potency as determined by this assay method indicates that degradation of the side chain at the 17-position has occurred. The paper chromatographic method (8) is specific for the intact steroid molecule being determined but has less precision than the other three methods.

**Fade-Ometer Tests.**—The solubilized steroid in aqueous solution was filled into vials and closed with the usual dropper fittings. Unexposed samples served as controls and provided the initial assay values. Some vials were wrapped in black paper and exposed to the ultraviolet light. These served as

controls for the effect of heat. Unlabeled flint amber, or polyethylene vials were used as test specimens. A "wrap-around" commercial label was placed on other flint and polyethylene vials to test the protection afforded by this type of label. The various samples were exposed for six hours in a Type FDA-R Fade-Ometer manufactured by the Atlas Electric Devices Co., Inc., Chicago, Ill. Exposed and unexposed samples were assayed by the ultraviolet, tetrazolium, and paper chromatographic (papergram) methods.

**Mercury Vapor Lamp Irradiation.**—Samples of hydrocortisone in an aqueous solution of Triton WR1339 (eye drops Optef, 0.2%<sup>2</sup>) were packaged in flint and amber vials. A 100 watt, Type H4 G E mercury vapor lamp was mounted two inches from the samples to serve as a light source. After irradiation for specified times the samples were removed and assayed by the ultraviolet, tetrazolium, and papergram methods.

**Miscellaneous Tests.**—Samples of hydrocortisone in an aqueous solution of Triton WR1339 were exposed in unlabeled flint and amber vials to diffuse

TABLE I.—THE EFFECT OF ULTRAVIOLET LIGHT ON SOLUBILIZED HYDROCORTISONE IN EYE DROPS OPTEF, 0.2%, CONTAINED IN UNLABELED FLINT VIALS

Light Source	Duration of Exposure	Concentration of Steroid, mg/cc		
		Ultraviolet	Tetrazolium	Papergram
Fade-Ometer	0 hr.	2.07	1.98	2.18
	6 hr.	1.78	1.78, 1.93	1.54
Mercury vapor lamp	0 hr.	2.06	2.13	2.23
	6 hr.	1.91	1.88	2.04
	22 hr.	1.54	1.87	1.75
Diffuse light	3 days	2.23	2.12	2.10
	7 days	2.18	1.98	2.03
	14 days	2.01	2.00	2.00
Sunlight	1 day	2.12	2.02	1.89

TABLE II.—THE EFFECT OF ULTRAVIOLET LIGHT ON SOLUBILIZED PREDNISOLONE IN EYE DROPS NEO-DELTEC, 0.2%, CONTAINED IN UNLABELED POLYETHYLENE VIALS

Light Source	Duration of Exposure, hr.	Concentration of Steroid, mg/cc		
		Ultraviolet	Tetrazolium	Papergram
Fade-Ometer	0	2.17	2.14	1.67 <sup>a</sup> , 1.8 <sup>a</sup> , 2.01 <sup>b</sup>
	6	0.586	2.25	0.20 <sup>a</sup> , 0.20 <sup>a</sup> , 0.24 <sup>b</sup>

\* Received October 23, 1959, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

<sup>1</sup> Registered trademark of Rohm and Haas Co.

<sup>2</sup> Registered trademarks of The Upjohn Co.

<sup>a</sup> Samples were stored for four months at room temperature before assay at two dilutions.

<sup>b</sup> Samples were stored for three months at 5°, then one month at room temperature before being assayed.



TABLE III.—RETARDATION OF PHOTOLYTIC DEGRADATIVE REACTIONS BY USE OF BLACK-WRAPPING, FLINT OR POLYETHYLENE VIALS WITH WRAP-AROUND LABELS, AND AMBER VIALS

Steroid	Light Source	Duration of Exposure	Type of Packaging	Concentration of Steroid, mg./cc.		
				Ultra-violet	Tetra-zolium	Papergram
Hydrocortisone	Fade-Ometer	6 hr.	Black-wrapped flint	2.07	1.98	2.25
Hydrocortisone	Fade-Ometer	6 hr.	Unlabeled amber	2.06	1.97,	2.25
					1.98	
Hydrocortisone	Fade-Ometer	6 hr.	Labeled flint	2.10	2.07	...
Hydrocortisone	Fade-Ometer	6 hr.	Unlabeled amber	2.13	2.21	2.25
Hydrocortisone	Fade-Ometer	22 hr.	Unlabeled amber	2.29	2.13	2.27
Hydrocortisone	Diffuse light	3 days	Unlabeled amber	2.16	2.24	2.15
Hydrocortisone	Diffuse light	7 days	Unlabeled amber	2.13	2.16	2.14
Hydrocortisone	Diffuse light	14 days	Unlabeled amber	2.07	2.14	2.02
Hydrocortisone	Sunlight	1 day	Unlabeled amber	2.18	2.20	2.08
Prednisolone	Fade-Ometer	6 hr.	Black-wrapped polyethylene	2.17	2.14	1.73 <sup>a</sup> , 1.72,
						1.48 <sup>b</sup>
Prednisolone	Fade-Ometer	6 hr.	Labeled polyethylene	1.73	2.14	0.91, <sup>a</sup> 0.98,
						1.13 <sup>b</sup>

a, b See footnotes to Table II.

light from a window (northern exposure) for three, seven, and fourteen days, and to sunlight for one day. In the latter case the exposure was approximately equivalent to six hours in direct sunlight. Inclement weather prevented controlled exposure for longer time intervals.

Solutions containing 40 mg./ml. of hydrocortisone, prednisolone, or methylprednisolone (Mcdrol<sup>2</sup>) in 95% ethanol were stored in 25-ml. volumetric flasks at room temperature (approximately 25°) under irradiation from ordinary fluorescent lighting<sup>3</sup> in the laboratory. Aliquots of the solutions were withdrawn after storage for specified times and assayed by the isonicotinic acid hydrazide and/or ultraviolet spectrophotometric methods.

RESULTS

Results of the exposure of solutions of hydrocortisone and prednisolone in aqueous solutions of Triton WR1339 to the various sources of ultraviolet light are given in Tables I, II, and III.

Results of the exposure of alcoholic solutions of hydrocortisone, prednisolone, and methylprednisolone to ordinary fluorescent laboratory lighting are given in Fig. 1. The slopes of the first order plots and the half-lives of the steroids under the conditions of the tests are given in Table IV.

DISCUSSION

Hydrocortisone in an aqueous solution of Triton WR1339 was found to undergo degradation when exposed to ultraviolet light in the Fade-Ometer only when the solution was packaged in unlabeled flint vials. A suitable "wrap-around" commercial label on the flint vial or an amber vial prevented degradation under the severe conditions of the test. The degradation which occurred in the unlabeled flint vial involved the A-ring as evidenced by the decrease in the U. V. assay. The results of exposing the aqueous hydrocortisone solution to the mercury vapor lamp confirmed this observation. Condition of exposure of the aqueous solutions of

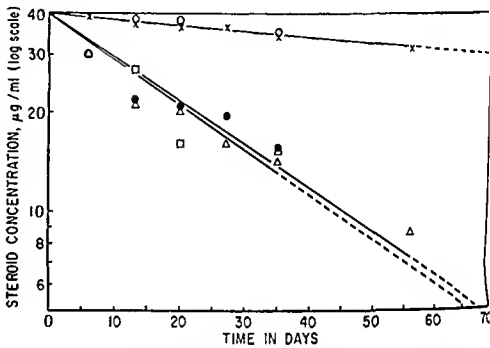


Fig. 1.—First order plots of the photolytic degradation of steroids when exposed to laboratory fluorescent lighting. Hydrocortisone: X, INF assay; O, U. V. assay. Prednisolone: Δ, INF assay; □, U. V. assay. Methylprednisolone: ●, INF assay.

TABLE IV.—SLOPES (*k*<sub>1</sub>)<sup>a</sup> AND THEIR STANDARD DEVIATIONS (*S*<sub>k1</sub>) OF FIRST ORDER PLOTS, SHOWN IN FIG. 1, FOR PHOTOLYTIC DEGRADATION OF STEROIDS IN 95% ALCOHOL<sup>b</sup>

Steroid	10 <sup>3</sup> <i>k</i> <sub>1</sub>	10 <sup>4</sup> <i>S</i> <sub>k1</sub>	<i>t</i> <sub>1/2</sub> <sup>c</sup> days
Hydrocortisone	0.188	0.944	160
Prednisolone	1.330 <sup>d</sup>	8.304	22.6 <sup>e</sup>
Methylprednisolone	1.386 <sup>d</sup>	12.78	21.7 <sup>e</sup>

<sup>a</sup> The slope or rate constant, *k*<sub>1</sub>, is in units of (2.303 days)<sup>-1</sup>  
<sup>b</sup> Solutions stored in 25-ml. volumetric flasks at room temperature (25°) under irradiation from the ordinary fluorescent laboratory lighting.  
<sup>c</sup> The half-life of the steroid calculated by means of the expression *t*<sub>1/2</sub> = log<sub>10</sub> 2/*k*<sub>1</sub>.  
<sup>d</sup> The slopes do not differ significantly.  
<sup>e</sup> The half-lives do not differ significantly.

<sup>2</sup> A laboratory approximately 16 × 22 feet was lighted by four fluorescent lights, each of which contained three 40-inch, 40-watt fluorescent tubes and which were mounted about 9 feet above the floor. Almost the entire northeast wall was glass. The samples were stored on a shelf two feet below and about two and one-half feet from one end of one of the fluorescent lights.

hydrocortisone to ultraviolet light in the Fade-Ometer and to the mercury vapor lamp were much more drastic than may be expected from exposure to diffuse light or even direct sunlight.

Prednisolone in aqueous solution of Triton WR1339 was photolytically degraded to a greater extent than hydrocortisone when exposed in the Fade-Ometer. The degradation of prednisolone, caused by the ultraviolet light, appeared to involve only the A-ring since the ketol side chain remained

intact as indicated by the tetrazolium assays for the exposed samples compared with the controls.

The results obtained with the labeled polyethylene and flint vials in this study are valid only for the type of "wrap-around" commercial label utilized. If the label were smaller and did not cover as much of the surface area of the vial, equivalent results would not be expected. It is interesting from a pharmaceutical viewpoint that protection against photolytic degradation can be obtained by the use of a suitable label as well as by the use of amber glass. Additional protection against ultraviolet light is afforded by the cardboard carton which covers the vial except when the product is actually in use.

Ordinary fluorescent laboratory lighting causes photolytic degradation of hydrocortisone, prednisolone, and methylprednisolone as indicated by the results in Fig. 1. The slopes of the first order plots for the degradation of prednisolone and methylprednisolone were not significantly different but the slope for each of these steroids was 7 to

7.5 times the slope for hydrocortisone under the same test conditions. Hence, the two double bonds present in prednisolone and methylprednisolone make these steroids much more susceptible to ultraviolet light than the one double bond in the A-ring of hydrocortisone. Standard alcoholic solutions of these steroids should be stored in amber bottles or black-wrapped flint bottles at refrigerator temperature to ensure maintenance of the potency of the solutions.

#### REFERENCES

- (1) Savard, K., Wotiz, H. W., Marcus, P., and Lemon, H. M., *J. Am. Chem. Soc.*, **75**, 6327(1953).
- (2) Barton, D. H. R., and Taylor, W. C., *ibid.*, **80**, 244 (1958).
- (3) Belgian pat. 564,254 (1959).
- (4) Johnson, R. H. (to The Upjohn Co.), U. S. pat. 2,880,138 (1959).
- (5) Guttman, D. E., Hamlin, W. E., Shell, J. W., and Wagner, J. G., *THIS JOURNAL*, "Manuscript in preparation".
- (6) Umberger, E. J., *Anal. Chem.*, **27**, 768(1955).
- (7) Wagner, J. G., Dale, J. K., Schlager, C. A., Meister, P. D., and Booth, R. E., *THIS JOURNAL*, **47**, 580(1958).
- (8) Meister, P. D., Schlager, C. A., Stafford, J. E., and Johnson, J. L., *ibid.*, **47**, 576(1958).

## A Note on an Infrared Assay of Chloroform in Pharmaceutical Products\*

By JOHN C. SOUDER and PATRICK DELUCA†

A quantitative infrared spectrophotometric assay for chloroform in pharmaceutical preparations has been developed. The procedure, which is sufficiently accurate for the control of filling operations, consists of extraction with carbon disulfide followed by estimation of chloroform by its characteristic infrared absorbance at 13.17 microns. Reproducibility of the method falls within  $\pm 2.9$  per cent of the average.

is unusually strong and it occurs in a relatively sparse region of the spectrum. Chloroform concentrations of 4 to 12 mg. per ml. of carbon disulfide were found to be useful in 0.1-mm. absorption cells. Carbon disulfide is quite transparent at 12 to 14  $\mu$  and thicker absorption cells could readily be used for lower chloroform concentrations.

#### EXPERIMENTAL

**Preparation of Standards.**—Standards were prepared by weighing known quantities of chloroform into 50-ml. volumetric flasks containing 40 to 45 ml. of ethyl alcohol. The flasks were then diluted to volume with ethyl alcohol. Volumetric aliquots of the alcohol-chloroform solution containing 100 to 300 mg. of chloroform were added to 75 ml. of distilled water in separatory funnels. The water-alcohol-chloroform standards were then extracted with 2–10 ml. and 1–5 ml. volumes of carbon disulfide, collecting the extracts in 25-ml. volumetric flasks. These carbon disulfide extracts are dry enough to use directly in infrared absorption cells without additional drying. A Perkin-Elmer model 21 infrared spectrophotometer equipped with sodium chloride optics was used for all the work reported here.

The absorption difference between 13.17  $\mu$  and 12.5  $\mu$  was used as an indication of chloroform content (Fig. 1).

Pharmaceutical samples were extracted and determined in the same way. Five different chloroform-containing liquid preparations were selected at random and extracted as described above for

**T**HE USUAL PROCEDURES for determining chloroform are based upon hydrolysis and titration (2, 4, 6, 8) or the colorimetric reaction of Fujiwara (1, 3, 5, 7). These procedures are somewhat time consuming when used to control the filling of pharmaceutical products containing chloroform.

Chloroform is an ideal material for infrared spectrophotometric estimation. Chloroform is easily extracted from aqueous solutions into water-immiscible solvents such as carbon disulfide or cyclohexane which are suitable for use in infrared spectrophotometry. Carol (9) reported infrared determination of chloroform in drugs based on the 8.29  $\mu$  absorption maximum as the wavelength of choice using carbon disulfide as a solvent. In the present study the carbon-chlorine stretching band at 13.17  $\mu$  was used. The absorption peak at 13.17  $\mu$  was selected for quantitative estimation because it

\* Received August 21, 1959, from Smith Kline and French Laboratories, Philadelphia 1, Pa.

† Present address, Temple University, School of Pharmacy, Philadelphia, Pa.

Presented to the Scientific Section, A. Ph. A. Cincinnati meeting, August 1959.

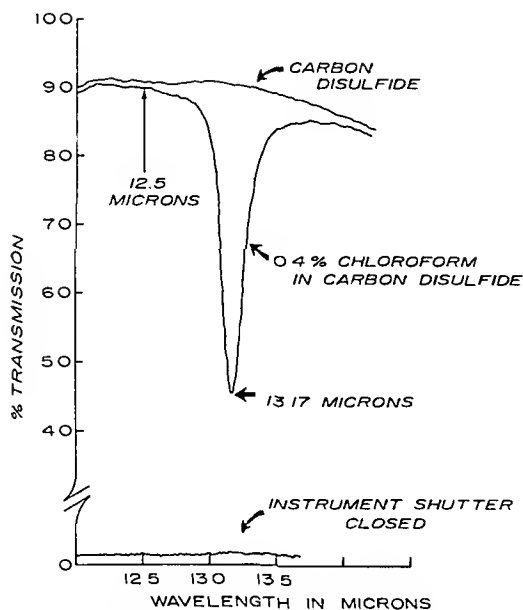


Fig. 1.—Infrared absorption of chloroform in carbon disulfide between 12 and 14  $\mu$ .

preparation of standards. In working with both standards and pharmaceutical preparations, it was necessary to force liquid into pipets with pressure rather than vacuum to avoid the loss of chloroform. Aliquots representing 100 to 250 mg. of chloroform were diluted 1:1 with water to make them more fluid and extracted with carbon disulfide. Only one of the five products had to be distilled before extraction to prevent emulsification. This one sample was diluted 1:1 with water and distilled through a water-cooled condenser into 25 ml. of alcohol cooled in an ice bath.

The same products were also assayed by alkaline hydrolysis and titration. Chloroform was hydrolyzed for titration by heating with 5 *N* alcoholic potassium hydroxide in pressure bottles for one hour in a boiling water bath. After hydrolysis, the solution was acidified with nitric acid and titrated potentiometrically with 0.1*N* silver nitrate solution. A comparison of results obtained by the two methods is shown in Table I. Three of the

TABLE I.—CHLOROFORM ASSAY RESULTS BY INFRARED AND HYDROLYSIS-TITRATION PROCEDURE

Product	Chloroform Found By Infrared Assay, mg./50 ml.	Chloroform Found By Alkaline Hydrolysis and Titration, mg./50 ml.
A	233.6	239.2
B	251.0	242.0
C	86.3	87.7
D	143.4	134.6
E	78.6	74.6

TABLE II.—REPRODUCIBILITY OF INFRARED CHLOROFORM ASSAY

Chloroform Assays in mg./50 ml.	
Run 1	Run 2
137.1	137.2
136.2	136.3
136.2	135.5
134.5	133.2

Average = 135.8 mg./50 ml.  
Standard Deviation ( $\sigma$ ) = 1.3 mg./50 ml.  
 $3 \times$  Standard deviation = 3.9 mg./50 ml. or  $\pm 2.9\%$ .

five samples required distillation to prevent interference when assayed by hydrolysis and titration.

Reproducibility of the infrared method was tested by running two groups of four aliquots from a single sample. The results obtained are shown in Table II. Standard deviation was found to be 1.3 mg./50 ml. or about 1%. For this same run of eight samples, the reproducibility ( $3 \times$  S. D.) was  $\pm 2.9\%$ .

#### REFERENCES

- (1) Feldstein, M. and Klendshoj, N. C., *Can. J. Med. Technol.*, **17**, 127(1955).
- (2) Stainier, C., and Grosjean, L., *Ind. Chim. Belge*, **20**, Spec. No., 551(1955).
- (3) Sasaki, D., *Nisshin Igaku*, **42**, 344(1955).
- (4) Kiba, T., and Terada, K., *J. Chem. Soc. Japan, Pure Chem. Sect.*, **75**, 196(1954).
- (5) Kubalski, J., *Acta Polon. Pharm.*, **10**, 269(1953); **11**, 39(1954).
- (6) Hanna, J. G., and Siggia, S., *Anal. Chem.*, **22**, 569(1950).
- (7) Burgen, A. S. V., *Brit. Med. J.*, **1**, 1238(1948).
- (8) Gauzer, E., and Pashaeva, K., *Mysnaya Ind.*, **S. S. S. R.**, **19**, 82(1948).
- (9) Carol, J., *J. Assoc. Offic. Agr. Chemists*, **38**, 628(1955).

# A Note on the Occurrence of 2,6-Dimethoxybenzoquinone in *Rauwolfia vomitoria*\*

By S. MORRIS KUPCHAN and MANG E. OBASI

**R**AUWOLFIA VOMITORIA Afz. originates in Africa in the region from upper Guinea and Uganda to Angola and Nyasaland (1). Roots of *R. vomitoria* have been shown to contain the important hypotensive and sedative alkaloids reserpine and rescinnamine, as well as several other indole alkaloids, and the roots of this Apocynaceous plant represent an important commercial source of reserpine (1).

The work reported herein concerns a nonalkaloidal constituent of an alkaloid-rich extract of *R. vomitoria*. The extraction procedure consisted of extracting, with chloroform the powdered *R. vomitoria* root, previously wetted with water. The chloroform extracts were evaporated to dryness and the residue was dissolved in methanol-acetic acid. On standing, a brown solid separated in crystalline form (ca 0.002% based on root).<sup>1</sup>

Recrystallization of the crude brown crystalline solid from dioxane afforded light yellow needles, m. p. 254–255°. Analysis of the compound afforded results which supported a  $C_8H_8O_4$  formula. Reduction with sodium bisulfite gave a dihydro derivative,  $C_8H_{10}O_4$ , which reverted to the original compound upon exposure at room temperature to air oxidation. Bromination in carbon disulfide afforded a dibromo derivative,  $C_8H_6Br_2O_4$ , and reductive acetylation gave a diacetate,  $C_{12}H_{14}O_6$ . The foregoing characteristics led to identification of the compound as 2,6-dimethoxybenzoquinone, and the identification was confirmed by direct comparison with an authentic synthetic sample prepared by oxidation of pyrogallol trimethyl ether (2,3).

The isolation of 2,6-dimethoxybenzoquinone from *Adonis vernalis* of the family Ranunculaceae was reported some years ago (4). 2,6-Dimethoxybenzoquinone has been shown to exert strong bacteriostatic activity against a variety of microorganisms (5–8). One might speculate that the teleological significance of the occurrence of 2,6-dimethoxybenzoquinone in the plant kingdom may be associated with the bacteriostatic activity; perhaps this compound plays a role in the defense of plants against pathogens.

## EXPERIMENTAL

Melting points are corrected for stem exposure. Infrared spectra were determined on a Baird model B double beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined on a model 11 MS Cary recording spectrophotometer and chloroform was used as solvent.

\* Received December 11, 1959, from the Department of Pharmaceutical Chemistry, University of Wisconsin, Madison.

Supported in part by a grant from the National Institutes of Health [H-2952 (C2)].

<sup>1</sup> The isolation and preliminary purification was done by Riker Laboratories, Inc., Northridge, Calif. We gratefully acknowledge the generosity of Mr. Murle W. Klohs in forwarding to us the crude crystalline material and information concerning its physical constants.

Microanalyses were carried out by Drs. Weiler and Strauss, Oxford, England.

**Purification of 2,6-Dimethoxybenzoquinone Derived from *Rauwolfia vomitoria*.**—The brown crystalline solid obtained from methanol-acetic acid in the extraction procedure was recrystallized twice from dioxane with Norite treatment. Light yellow needles were obtained, m. p. 254–255°. The analytical sample was further purified by sublimation.

**Anal.**—Calcd. for  $C_8H_8O_4$ : C, 57.15; H, 4.80; OMe, 36.92. Found: C, 56.93; H, 4.70; OMe, 37.38.

**Reduction of the Naturally-Derived 2,6-Dimethoxybenzoquinone.**—A suspension of 2,6-dimethoxybenzoquinone (400 mg.) in water (5 cc.) was heated on the steam bath and treated dropwise with 20% sodium bisulfite solution (3.5 cc.). The yellow solid dissolved to form a colorless solution. Upon cooling, a colorless crystalline solid separated (250 mg.). Recrystallization from water afforded 2,6-dimethoxyhydroquinone in the form of colorless needles (200 mg.), m. p. 163–164°. [Reported m. p. 160° (4)].

**Anal.**—Calcd. for  $C_8H_{10}O_4$ : C, 56.45; H, 5.92; OMe, 36.47. Found: C, 56.20; H, 5.95; OMe, 38.45.

A solution of the hydroquinone in 95% alcohol was allowed to stand at room temperature overnight. A brown crystalline solid separated which afforded pure 2,6-dimethoxybenzoquinone, m. p. 254–255°, after recrystallization from dioxane.

**Reductive Acetylation of the Naturally-Derived 2,6-Dimethoxybenzoquinone.**—To a suspension of 2,6-dimethoxybenzoquinone (500 mg.) in acetic anhydride (2.5 cc.) was added zinc powder (1.0 Gm.) and powdered anhydrous sodium acetate (100 mg.). The mixture was heated gently until the yellow color disappeared, and then boiled for two minutes. The hot supernatant solution was decanted from the inorganic residue; the residue was washed with hot, glacial acetic acid (4 cc.) and the washings were added to the supernatant. The combined solution was heated, treated carefully with water to decompose excess acetic anhydride, and then further to turbidity. Upon cooling, 2,6-dimethoxyhydroquinone diacetate separated in the form of colorless crystals (525 mg.), m. p. 125–127°. Recrystallization from dilute alcohol gave colorless needles, m. p. 127–128°. [Reported m. p. 129–129.5° (9)].

**Anal.**—Calcd. for  $C_{12}H_{14}O_6$ : C, 56.70; H, 5.55. Found: C, 55.87; H, 5.24.

**Bromination of the Naturally-Derived 2,6-Dimethoxybenzoquinone.**—To a suspension of 2,6-dimethoxybenzoquinone (350 mg.) in carbon disulfide (5 cc.) was added excess bromine in carbon disulfide, and the solution was allowed to stand at room temperature overnight. Evaporation to dryness with a gentle current of air left a red crystalline residue. Recrystallization from absolute alcohol with Norite treatment gave 2,6-dimethoxy 3,5-dibromobenzoquinone in the form of red

feathery plates (270 mg.), m. p. 177–178°. [Reported m. p. 175° (10)].

*Anal.*—Calcd. for  $C_6H_6Br_2O_4$ : C, 29.48; H, 1.86; Br, 49.03. Found: C, 30.06; H, 1.77; Br, 48.75.

**Synthesis of 2,6-Dimethoxybenzoquinone.**—2,6-Dimethoxybenzoquinone, m. p. 254–255°, was synthesized by oxidation of pyrogallol trimethyl ether with nitric acid in ethanol (2, 3). The melting point was not depressed upon admixture with the naturally-derived sample. Infrared and ultraviolet spectral comparison of the respective samples afforded positive proof of identity.

## REFERENCES

- (1) Woodson, R. E., Youngken, H. W., Schlittler, E., and Schneider, J. A., "Rauwolfia: Botany, Pharmacognosy, Chemistry, and Pharmacology," Little, Brown and Company, Boston, Mass., 1957.
- (2) Graebe, von C., and Hess, H., *Ann.*, **340**, 232 (1905).
- (3) Baker, W., *J. Chem. Soc.*, **1941**, 665.
- (4) Karrer, W., *Helv. Chim. Acta*, **13**, 1424 (1930).
- (5) Oxford, A. E., *Chem. & Ind.*, **61**, 189 (1942).
- (6) Wiedling, S., *Acta Pathol. Microbiol. Scand.*, **22**, 379 (1945).
- (7) Hoffmann-Ostenhof, O., and Fellner-Feldegg, H., *Monatsh.*, **80**, 720 (1949).
- (8) Rao, K. R., and Seshadri, T. R., *J. Sci. Ind. Research India*, **14C**, 189 (1955).
- (9) Huismans, H. O., *Rec. trav. chim.*, **69**, 1133 (1950).
- (10) Hunter, W. H., and Levine, A., *J. Am. Chem. Soc.*, **48**, 1608 (1926).

## Book Notices

**British Pharmaceutical Codex 1959.** Published by direction of the Council of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, 17, Bloomsbury Square, London, W. C., England, 1959. Available in the U. S. from Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia 3, Pa. xxix + 1,301 pp. 14.5 x 22.5 cm. Price 70s.

This seventh edition of the *British Pharmaceutical Codex* (first B. P. C. in 1907) carries on the aim of providing information on drugs in common use throughout the British Commonwealth. Included are many drugs, not official in the "British Pharmacopoeia," for which tests and standards have been developed.

Inclusion of many drugs that have been developed since the 1954 edition of the B. P. C. has been offset by deletions and revisions to the extent that B. P. C. 1959 has forty pages less than B. P. C. 1954. Monographs on official drugs indicate that the substance must comply with the requirements of the "British Pharmacopoeia." All monographs include statements on actions and uses, and give dosage forms along with a brief descriptive note on each preparation. Dosage forms that are not in the B. P. C. are given in detail in a "Formulary" section of the B. P. C. Thus, under calamine in the "General Monographs" section, there are given calamine lotion B. P. and four unofficial preparations of calamine. The latter four preparations are treated more fully in the B. P. C. "Formulary" section, giving the formulas, procedures for preparation, and standards.

The policy governing admissions of drugs into B. P. C. 1959 is the same as for B. P. C. 1954. This was discussed in the review of B. P. C. 1954 in *THIS JOURNAL*, **44**, 190 (1955). It is very similar to the admissions policy for our "National Formulary." Seventy new monographs have been added to the "General Monograph" section and 79 dosage forms (45 of which were in B. P. 1953 but not in B. P. 1958) have been added to the "Formulary" section. Deleted were 99 general monographs (including leech) and 120 formulary monographs.

Three new appendixes (XIV, XV, and XVI) deal

with milliequivalents to assist in the preparation of parenteral infusion solutions, biological assays for chloramphenicol eye ointment and for neosarsphenamine, and limits on diameters of tablets.

The Codex Revision Committee has performed its task well in preparing this edition of a valuable reference book for pharmacy.

**Organic Syntheses**, Vol. 39. Edited by MAX TISLER. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1959. 114 pp. 15 x 23 cm. Price \$4.

This volume includes methods of preparation for: N-(*p*-acetylaminophenyl)rhodanine, benzenboronic anhydride, 2,5-diamino-3,4-dicyanothiophene, *di-n*-butyldivinyltin, dicyanoketene ethylene acetal, 9,10-dihydroxystearic acid, N,N-dimethylcyclohexylmethylamine, *p*-dithiane, ethyl (1-phenylethylidene)cycanoacetate, indazole, indole-3-aldehyde, 2-mercapto-4-amino-5-carbethoxypyrimidine and 2-mercapto-4-hydroxy-5-cyanopyrimidine, methyl cyclopentanecarboxylate, methylenecyclohexane and N,N-dimethylhydroxylamine hydrochloride, 9-methylfluorene, 3-methyl-2-furoic acid and 3-methylfuran, methyl 3-methyl-2-furoate,  $\beta$ -methyl- $\beta$ -phenyl- $\alpha,\alpha'$ -dicyanoglutarimide,  $\beta$ -methyl- $\beta$ -phenylglutaric acid, 1-phenyl-1-penten-4-yn-3-ol, phenylpropargyl aldehyde diethyl acetal, tetracyclotetane, tetracyanoethylenic, *p*-tricyanovinyl-N,N-dimethylaniline, 2,4,4-trimethylcyclopentanone,  $\alpha,\alpha,\beta$ -triphenylpropionitrile, triptycene, and trithiocarbodiglycolic acid. A cumulative subject index for volumes 30–39 in this series is appended.

**Steric Course of Microbiological Reactions.** By Ciba Foundation Study Group No. 2. Little, Brown & Co., 34 Beacon St., Boston 6, Mass., 1959. vii + 115 pp. 12 x 18.5 cm.

A compilation of the papers and discussions at the symposium of the Ciba Foundation study group No. 2, March 17, 1959. References and an index are appended.

JOURNAL OF THE  
AMERICAN PHARMACEUTICAL  
ASSOCIATION

VOLUME 49

MAY 1960

NUMBER 5

The Synthesis and Antifungal Studies of Some  
Benzofuran Compounds\*

By FOO PAN† and GAIL A. WIESE

An improved synthesis of benzofuran-2-carboxaldehyde and 2-methylbenzofuran-3-carboxaldehyde and the preparation of twenty-three new derivatives are reported. They were evaluated against three pathogenic fungi, together with some other derivatives which have been described in the literature. Some of these compounds showed significant *in vitro* antifungal activity. Of all the compounds tested, including the standard, undecylenic acid, benzofuran-2-carboxaldehyde exhibited high activity in every case against all three organisms. A discussion of the activity and structure relationship is based on the data resulting from this investigation. Further studies seem to be warranted.

SOME ALDEHYDES and their derivatives have been found to be effective antifungal and/or antibacterial agents. Powerful antifungal as well as antibacterial activity of decanoylacetaldehyde and related compounds was reported by Noguchi and Okeda (1). Halo-2,3-butanedione-3-oximes have been patented as antifungal agents (2) and some monosubstituted salicylaldehyde alkoximes (3) were found to be effective as fungicides in dilutions as low as one p. p. m. Ward and co-workers (4) reported the outstanding fungistatic activity of 5-nitro-2-furfural oxime and a few other nitrofurans compounds. 5-Nitro-2-furfural semicarbazone (5) has been successfully used for the treatment of various dermatological conditions and N-(5-nitro-2-furfurylidene)-1-aminohydantoin (6) has also been established as an antibacterial agent.

Benzofuran compounds remain uninvestigated as antifungal agents, probably due to the fact that so far no benzofuran compound has found

application in pharmaceutical chemistry (7). It is interesting to note, however, that griseofulvin, an orally effective (8) antifungal antibiotic, is a carbonyl derivative of 2,3-dihydrobenzofuran (9) and that some derivatives of thianaphthene (10), a sulfur analog of benzofuran, have been found to possess considerable antifungal activity.

In view of the facts outlined above, two benzofurancarboxaldehydes and a number of their derivatives have been synthesized and screened *in vitro* for their antifungal activity against *Microsporum audouinii*, *Microsporum gypseum*, and *Trichophyton rubrum*, by agar-plate, paper-disk technique. Some of the more active and the less soluble compounds were tested in the form of ointment. Undecylenic acid was used in both instances as a standard.

The compounds prepared and tested are shown in Table I. The results of the antifungal studies are listed in Tables II and III.

All 28 derivatives, including 23 new compounds, were prepared by condensation reactions involving the aldehyde group. The condensing agents included various aromatic and heterocyclic amines, isoniazid, aminoguanidine, and acetone, as well as the usual characteristic

\* Received August 13, 1959, from the State University of Iowa, College of Pharmacy, Iowa City

Abstracted from a thesis submitted by Foo Pan to the Graduate College of the State University of Iowa in partial fulfillment of the requirements for the degree of Master of Science, August 1959

† Fellow of the China Medical Board of New York, Inc., 1955-1959. Present address: Department of Biochemistry, National Defense Medical Center, Taipei, Taiwan, China.

TABLE I.—BENZOFURAN COMPOUNDS SYNTHESIZED AND TESTED

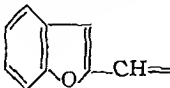
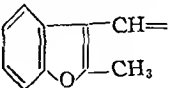
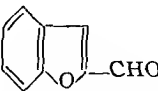

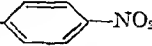
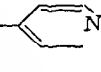
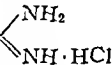

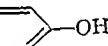
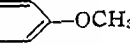
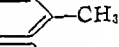
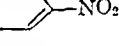
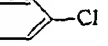


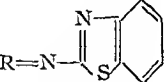
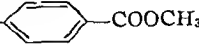
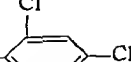
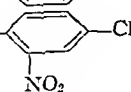
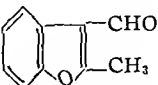

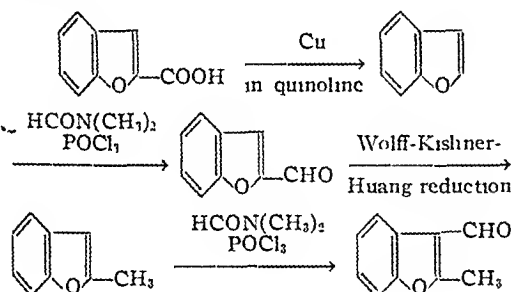
		$R = $ 	$R' = $ 				
No.	Structure	Appearance	M. p., ° C. <sup>a</sup>	Yield, %	Nitrogen, % Calcd.	Found	Reference <sup>c</sup>
I		...	...	..	...	...	12, E
II	$R=O \cdot NaHSO_3$	...	...	..	...	...	13, E
III	$R=NNH-$ 	...	...	..	...	...	14
IV	$R=NNHCONH_2$	...	...	..	...	...	14
V	$R=NNHC(SNH_2)$	...	...	..	...	...	13
VI <sup>d</sup>	$R=NNH-$ 	Brownish red needles	213-214	73	14.94	14.88	E
VII <sup>d</sup>	$R=NOH$	White needles	131-132	80	8.69	8.64	E,
VIII <sup>d</sup>	$R=NNHC-$ 	Colorless prisms	200-202	71	15.85	15.71	E
IX <sup>d</sup>	$R=NNHC-$ 	White crystals	176-178	15	23.48	23.73	E
X <sup>d</sup>	$R=N-$ 	Pale yellow crystals powdered	56-56.5 <sup>e</sup>	46	6.33	6.17	E
XI <sup>d</sup>	$R=N-$ 	Bright yellow needles	230-231 (Decompn.)	42	5.91	5.79	E
XII <sup>d</sup>	$R=N-$ 	Yellow crystals powdered	89-90	65	5.58	5.49	E
XIII <sup>d</sup>	$R=N-$ 	Bright yellow fine crystals	95-96.5	62	5.96	5.82	E
XIV <sup>d</sup>	$R=N-$ 	Bright yellow crystals powdered	176-177	55	10.53	10.27	E
XV <sup>d</sup>	$R=N-$ 	Pale yellow needles	138-139.5	71	5.48	5.45	E
XVI <sup>d</sup>	$R=N-$ 	Pale yellow crystals powdered	109-111	23	12.61	13.12	E
XVII <sup>d</sup>	$R=N-$ 	Yellow crystals powdered	131-132	70	10.29	9.94	[E
XVIII <sup>d</sup>	$R=N-$ 	Pale yellow crystals powdered	138-139	36	10.08	10.24	E
XIX <sup>d</sup>	$R=CHCOCH_3$	Yellow needles	97-98 <sup>f</sup>	22 C; 77.42 H: 5.38	77.35 5.17	E	
XX <sup>d</sup>	$R=N-$ 	Shining yellow scales	133.5-134.5	70	5.02	5.00	E
XXI <sup>d</sup>	$R=N-$ 	Yellow crystals powdered	92-93	60	4.83	4.70	E
XXII <sup>d</sup>	$R=N-$ 	Reddish yellow needles	112-112.5	40	9.32	9.18	E
XXIII		...	...	..	...	...	11, E
XXIV <sup>d</sup>	$R'=N-$ 	Greenish yellow prisms	130-131	21	5.58	5.34	E

TABLE I (Continued)

No	Structure	Appearance	M p, ° C <sup>a</sup>	Yield, %	Nitrogen, <sup>b</sup> % Calcd	Found	Reference
XXV <sup>d</sup>		Bright yellow scales	77.5-78.5	46	5.19	5.04	E
XXVI <sup>d</sup>		Yellow crystals powdered	231-232 (Decompn)	56	18.01	17.43	E
XXVII <sup>d</sup>		Pale yellow needles	203-204	58	15.05	14.83	E
XXVIII <sup>d</sup>		Pale yellow needles	114-115	74	8.00	7.87	E
XXIX <sup>d</sup>		Bright yellow platelets	129-130	89	11.20	11.08	E
XXX							11

<sup>a</sup> Determined by capillary method and uncorrected <sup>b</sup> Carbon and hydrogen for Compound XIX <sup>c</sup> F, experimental.  
<sup>d</sup> New compound <sup>e</sup> Boiling point, 164°/1 mm <sup>f</sup> Boiling point, 128°/1 mm

reagents for carbonyl compounds. The process used for preparing the two benzofurancarboxaldehydes is indicated below. The procedure of Bisagni and co-workers (11) for the formulation of benzofuran was modified to give improved yields.



### EXPERIMENTAL

#### Synthesis<sup>1</sup>

**Benzofuran-2-carboxaldehyde(I)**—To a mixture of 37.5 Gm. of benzofuran, b p 59-61° (6 mm), prepared from coumarilic acid in 81% yield by a modified procedure of Tanaka (15), and 27.6 Gm. of N,N-dimethylformamide, 55.2 Gm. of phosphorus oxychloride was added in small portions with shaking, while the temperature was kept below 50°. The reaction mixture was allowed to stand at room temperature overnight. It was heated gradually to about 95° and then kept at 93 ± 2° for nine hours, with occasional shaking. It was again allowed to stand overnight. The mixture was poured into 500 Gm. of ice and water with stirring, and extracted with three 200-cc portions of ether. The ethereal extract was washed twice with 150-cc portions of 5% hydrochloric acid. These aqueous washings were in turn extracted with 100 cc of ether. The combined ethereal extracts were washed with a saturated sodium bicarbonate solution and then with water. When the ethereal solution was shaken in a mechanical shaker with a solution of 180 Gm. of sodium bisulfite in 300 cc of water, a white addition product of benzofuran-2-carboxaldehyde deposited.

The precipitate was collected on a filter and washed successively with alcohol and ether.

The aldehyde also may be obtained from the ethereal solution, after drying over anhydrous sodium sulfate and removing the solvent, by fractional distillation under reduced pressure. The yield of the free aldehyde, b p 90-93° (1 mm), *n*<sub>D</sub><sup>20</sup> 1.6334, or its sodium bisulfite adduct, was 67-71%.

**Benzofuran-2-carboxaldehyde *p*-Nitrophenylhydrazone (VI) and Benzofuran-2-carboxaldoxime (VII)**—Both of these compounds were prepared from 0.01 mole of the aldehyde by the usual method (16) and recrystallized from diluted alcohol.

**Benzofuran-2-carboxaldehyde Isonicotinoylhydrazone(VIII)**—A solution of 2.92 Gm (0.02 mole) of the aldehyde in 12 cc of alcohol was mixed with a solution of 2.75 Gm. of isomazid in 12 cc of alcohol and boiled for three minutes. The crystals which deposited after cooling were filtered and recrystallized from alcohol.

**Benzofurfurylideneaminoguanidine Hydrochloride(IX)**—A solution of 0.02 mole of benzofuran-2-carboxaldehyde in 50 cc of alcohol was mixed with 2.8 Gm. of aminoguanidine bicarbonate and 5.5 Gm. of sodium acetate. The mixture was heated on a water bath at about 60° for one hour, acidified to Congo red with concentrated hydrochloric acid, and filtered. The filtrate was concentrated to about one-half its original volume at room temperature and then cooled to about -10°. The precipitate which formed was collected and recrystallized three times from absolute alcohol.

**Benzofurfurylideneaniline(X)**—The aldehyde, 0.02 mole, was mixed with 2 cc of freshly redistilled aniline in a small evaporating dish. The mixture was heated on a boiling water bath for twenty minutes, cooled with stirring, and washed thoroughly with *n*-pentane. The solid was filtered and recrystallized from ether-*n*-pentane mixture.

**Benzofurfurylidene-*p*-aminophenol(XI)**—To a solution of 2.18 Gm. of *p*-aminophenol in 10 cc of absolute alcohol, 0.02 mole of benzofuran-2-carboxaldehyde was added with stirring. Reaction took place almost instantaneously. The mixture was heated under reflux for thirty minutes, cooled, and filtered. The product was recrystallized from methanol.

<sup>1</sup> Properties and yields of the new compounds prepared are shown in Table I.



TABLE II.—ANTIFUNGAL ACTIVITY OF SOLUTIONS OF BENZOFURAN COMPOUNDS<sup>a</sup>

Compound No. <sup>b</sup>	<i>M. audouinii</i>		<i>M. gypsum</i>		<i>T. rubrum</i>	
	1%	5%	1%	5%	1%	5%
Solution in Water						
II	0	1	0	9	3	12
Water	0	0	0	0	0	0
Solutions in Alcohol						
I	12	c	7	c	13	c
V	0	d	0		1	
VIII	0		0		0	
IX	5	11	3	5	2	15
X	0	4	2	5	1	10
XII <sup>c</sup>	0	0	0	0	5	0
XIII	0	0	0	0	0	1
XXIV	0	5	23	5	0	5
XXVIII	7	9	6	10	6	8
Undecylenic Acid	6	8	2	9	5	13
Alcohol	0	0	0	0	0	0
Solutions in Acetone						
III	0	0	1	1	1	2
VI	0	0	0	0	0	0
VII <sup>c</sup>	2	12	3	12	3	9
XI	0		0		0	
XV <sup>c</sup>	0	0	0	0	0	0
XVI <sup>c</sup>	0	5	12	1	17	5
XVII	0	0	0	0	0	2
XVIII	0		0		0	
XIX	2	5	1	5	3	6
XX	0		0		0	
XXI	0	1	0	2	0	5
XXII	8	5	12	6	5	10
XXIII	0	10	1	19	0	5
XXV	0	3	0	4	1	1
XXVI	0		0		0	
XXVII	0		0		0	
XXIX	3	5	8	1	7	3
Acetone	0	0	0	0	0	0

<sup>a</sup> Zones of inhibition in mm<sup>b</sup> The formulas of the compounds are listed in Table I, and their names in the experimental section<sup>c</sup> Little or no growth after 15-day incubation<sup>d</sup> Not tested<sup>e</sup> 4% solution instead of 5%

**Benzofurfurylidene-*p*-anisidine(XII) and Benzofurfurylidene-*p*-toluidine(XIII).**—These two compounds were prepared similarly to compound XI except that the heating time was two hours and the products were recrystallized from isopropyl alcohol

**Benzofurfurylidene-*p*-nitroaniline(XIV).**—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 20 cc. of absolute alcohol for four hours and the product recrystallized from benzene

**Benzofurfurylidene-*p*-chloroaniline(XV).**—To a solution of 0.02 mole of the aldehyde in 40 cc. of ether, 2.55 Gm. of *p*-chloroaniline was dissolved with stirring. Crystallization took place within a few minutes. After allowing to stand at room temperature for three hours, the mixture was filtered and the product recrystallized from alcohol

**Benzofurfurylidene-2-aminopyridine(XVI).**—Equimolar amounts (0.02 mole) of the aldehyde and 2-aminopyridine were heated under reflux in 5 cc. of absolute alcohol for four hours. The crystals formed after cooling were filtered, washed with ether, and recrystallized several times from diluted alcohol.

TABLE III.—ZONES OF INHIBITION IN MILLIMETI OF 5% OINTMENTS OF BENZOFURAN COMPOUND

Compound No. <sup>b</sup>	<i>M. audouinii</i>		<i>M. gypsum</i>		<i>T. rubrum</i>	
	1%	5%	1%	5%	1%	5%
I						
II	0		1		3	
IV	0		0		0	
V	0		0		2	
VII	5		8		9	
VIII	0		0		0	
IX	5		5		8	
X	3		4		8	
XI	0		0		1	
XIV	0		1		4	
XVI	1		12		8	
XVIII	2		1		5	
XX	0		1		2	
XXII	5		8		11	
XXIII	5		12		6	
XXIV	2		7		4	
XXVI	0		0		0	
XXVII	0		0		0	
XXVIII'	4		7		8	
XXX	0		0		1	
Undecylenic acid	4		3		8	
Hydrophilic ointment, U S P.	0		0		0	

<sup>a</sup> Only the more active and the less soluble compound were tested in ointment form<sup>b</sup> The formulas of the compounds are listed in Table I and their names in the experimental section<sup>c</sup> Little or no growth after 15-day incubation

**Benzofurfurylidene-3-aminoquinoline(XVII).**—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 10 cc. of absolute alcohol for four hours and the product recrystallized from diluted alcohol

**Benzofurfurylidene-2-aminobenzothiazole(XVIII).**—This compound was prepared in the same manner as compound XI, except the product was recrystallized from alcohol.

**Benzofurfurylideneacetone(XIX).**—To a mixture of 20 cc. of C. P. acetone and 0.01 mole of benzofuran-2-carboxaldehyde in a 250-cc., glass-stoppered reagent bottle, 2 cc. of 10% sodium hydroxide solution was added dropwise while the temperature was kept at 25–35°. The bottle was stoppered and shaken in a mechanical shaker at room temperature for three hours. The reaction mixture was acidified to litmus with diluted hydrochloric acid and the two layers were separated in a separatory funnel. The upper aqueous layer was extracted with 10 cc. of benzene and the benzene extract combined with the organic layer, washed with water, and dried over anhydrous sodium sulfate. After removal of the solvent, the product was purified by sublimation under reduced pressure.

**Methyl Benzofurfurylidene-*p*-aminobenzoate(XX).**—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 5 cc. of absolute alcohol for one hour. The crude product was washed with ether and recrystallized from methanol.

**Benzofurfurylidene-2,4-dichloroaniline(XXI).**—A mixture of 0.02 mole of the aldehyde and 3.24 Gm. of 2,4-dichloroaniline was heated under reflux in 5 cc. of absolute alcohol for two hours and then

cooled by ice and water. The crude product, deposited as a heavy oil layer, was separated from most of the solvent by decantation and dissolved in ether. Crystallization took place when the ethereal solution was stirred and cooled by a dry ice and acetone mixture. The crystals formed were collected on a filter and recrystallized from diluted alcohol.

**Benzofurfurylidene-2-nitro-4-chloroaniline (XXII).**

—This compound was prepared in the same manner as compound XI, except the reactants were heated under reflux in 5 cc. of absolute alcohol for two hours and the product recrystallized from dilute alcohol.

**2-Methylbenzofuran.**—This compound, b p 192–194°, was prepared in 85% yield by the procedure of Bisagni and co-workers (11), using 0.1 mole of benzofuran-2-carboxaldehyde, 50 cc. of diethylene glycol, 15 Gm. of 99–100% hydrazine hydrate, and 15 Gm. of potassium hydroxide.

**2-Methylbenzofuran-3-carboxaldehyde (XXIII).**—2-Methylbenzofuran (17.7 Gm) was formylated with N,N-dimethylformamide (10.6 Gm.) and phosphorus oxychloride (23 Gm.) by the procedure described for the preparation of benzofuran-2-carboxaldehyde except the reaction mixture was heated at  $93 \pm 2^\circ$  for eleven hours and the product isolated as the free aldehyde by concentrating the dried ethereal extract and recrystallizing the crystals from petroleum ether. The yield of the yellow needles, m. p. 80.0–80.5°, was 16 Gm. (75%).

**2-Methyl-3-benzofurfurylidene-*p*-aminophenol (XXIV).**—Three grams of 2-methylbenzofuran-3-carboxaldehyde and 2.1 Gm. of *p*-aminophenol were dissolved in 10 cc. of hot absolute alcohol and heated under reflux for one and one-half hours. The product, failing to crystallize after the reaction mixture was allowed to stand in a refrigerator overnight, was cooled with a dry ice and acetone mixture. The product which precipitated was recrystallized first from methanol and then from benzene.

**2-Methyl-3-benzofurfurylidene-*p*-chloroaniline (XXV).**—A solution of 2 Gm. of 2-methylbenzofuran-3-carboxaldehyde and 1.65 Gm. of *p*-chloroaniline in 14 cc. of absolute alcohol was heated under reflux for two hours and then allowed to stand in a refrigerator overnight. The filtered precipitate was recrystallized from alcohol.

**2-Methylbenzofuran-3-carboxaldehyde Isonicotinoylhydrazone (XXVII).**—This compound was prepared as the benzofuran-2-carboxaldehyde analog from 1.92 Gm. (0.012 mole) of 2-methylbenzofuran-3-carboxaldehyde and an equimolar amount of isoniazid.

**2-Methylbenzofuran-3-carboxaldehyde Thiosemicarbazone (XXVI), 2-Methylbenzofuran-3-carboxaldehyde Oxime (XXVIII), and 2-Methylbenzofuran-3-carboxaldehyde Phenylhydrazone (XXIX).**—These compounds were prepared by the usual procedure (16), using 0.015 mole of the aldehyde. Compounds XXVI and XXVIII were recrystallized from diluted alcohol, while compound XXIX was recrystallized from alcohol.

#### Antifungal Section

The compounds prepared were screened *in vitro* for antifungal activity against *Microsporum audouinii*, *Microsporum gypsum*, and *Trichophyton rubrum* in solutions and/or ointments, using undecylenic acid as a standard.

An agar-plate, paper-disk technique, similar to that reported by Goettseh and Wiese (10), was used for the testing of the compounds in solutions. Sabouraud's dextrose agar, pH 5.6, with the addition of 0.1% of Tween 80, was used as the culture medium. The compounds were tested in 5% and/or 1% solutions in acetone or alcohol, according to the individual solubilities. In a few cases, 4% solutions were used instead of 5%. Water was used as solvent in one case. Acetone and alcohol were tested as controls. Two disks, 12.7 mm. in diameter, were placed on each culture plate and duplicate plates were run in each case. The zones of inhibition were measured after the plates were incubated at room temperature (25–29°) for seven days. Averages of four readings are listed in Table II.

The same method was used for the preparation of culture plates when ointments instead of solutions were used for testing. Five per cent ointments of those compounds, which were less soluble in the solvents used or showed higher activity in solutions, in hydrophilic ointment U. S. P., with no preservative, were prepared and placed in tubes under aseptic conditions. Two streaks of the ointment, about 50 mm. in length and 100 mg. in weight, were placed separately on each plate. Two plates were used for each compound. The ointment base was included in testing as control. These plates were also incubated at room temperature for seven days. Averages of the zones of inhibition, measured as the minimum distance between the periphery of the ointment and the colony growth, are in shown Table III.

#### DISCUSSION

Benzofuran-2-carboxaldehyde has been prepared from 2-methylbenzofuran by oxidation with selenium oxide (12), from the glyoxal compound derived from coumarilic acid (14), and from coumarilic acid anilide (13). The last method has been tried and proved satisfactory only on a very small scale. For the preparation of this compound, the authors have modified the procedure of Bisagni and co-workers (11). By modifying both the reaction conditions and the process of separation and purification, the yield has been improved, with 71% of the theoretical yield obtained as compared to a reported 37% yield. The procedure for preparing 2-methylbenzofuran-3-carboxaldehyde (11) has been similarly modified.

2-Methylbenzofuran, the intermediate for 2-methylbenzofuran-3-carboxaldehyde, was prepared in 85% yield by the Wolff-Kishner-Huang reduction of benzofuran-2-carboxaldehyde according to the procedure of Bisagni and co-workers (11). The poor yield originally reported is doubtful.

The procedure for the decarboxylation of coumarilic acid reported by Tanaka (15) has been modified in two aspects with favorable results. First, only one-third the quantity of quinoline was used, and secondly, the product was isolated and purified directly by fractional distillation.

As to the synthesis of the new benzofuran compounds, no difficulty was encountered in the preparation of common derivatives of aldehydes such as oximes, hydrazones, and thiosemicarbazones by the usual methods.

There were considerable amounts of resinous by-

products formed, probably from the alkali-catalyzed polymerization, when benzofurfurylideneacetone (XIX) was prepared. No suitable solvent for satisfactory crystallization of this compound was found, so sublimation under reduced pressure was finally employed to purify the product. Perhaps this side-reaction could be inhibited to some extent by conducting the reaction at lower temperatures.

The difficulties encountered in purifying the aminoguanidine and aminopyridine derivatives (IX, XVI) account for the poor yields.

In the preparation of the aniline derivative (X), it was found imperative to obtain crystals from the reaction mixture by cooling, washing, and filtering before any further purification could be accomplished. Otherwise, a pure product could not be obtained even by vacuum distillation.

The results of the *in vitro* antifungal studies, as shown in Tables II and III, indicate that most of the compounds tested exhibited some activity against one or more strains of fungi. Generally, nine compounds (I, VII, IX, X, XVI, XXII, XXIII, XXIV, and XXVIII) showed equal or greater activity than undecylenic acid, which is a frequently used standard antifungal agent. Solubility of the individual compound seems to be one of the factors which influence the activity, since none of the least soluble compounds exhibited significant activity even in ointments. In this connection it is interesting to note that compound XI was practically inactive while its analog, compound XXIV, which was much more soluble, was among the most active compounds.

Of all the compounds tested, benzofuran-2-carboxaldehyde (I) was most active in every case. 2-Methylbenzofuran-3-carboxaldehyde (XXIII), although more active than undecylenic acid against two of the three organisms used, was less active than benzofuran-2-carboxaldehyde. Chemically they are different in two ways, i. e., the position of the aldehyde group and the presence of a methyl group in XXIII. It is thought that the methyl group greatly decreases the polarity of the carbonyl group, and probably this factor plays a more important role in decreasing its antifungal activity. Although no conclusion should be drawn without further studies in this field, the relationship between the activity and polarity of antifungal compounds have been noted in several other studies (17-19).

Until more of the related compounds are prepared and studied, it is difficult to say whether the benzofuran nucleus or the aldehyde group contributes more to antifungal activity, or if both are essential. Although the antifungal antibiotic, griseofulvin (8) is a carbonyl derivative of benzofuran, Ward and co-workers (4) noted no structural specificity in the nitrofuran compounds studied.

The activity shown by a number of other compounds tested is not necessarily due to reformation of the parent aldehyde, since among seven derivatives of compound XXIII studied, three compounds (XXIV, XXVIII, and XXIX) exhibited somewhat

greater activity than the parent compound in one or more cases.

Among the usual derivatives of the two aldehydes, both oximes are quite effective. This agrees with the fact that most of the functional derivatives of aldehydes reported to possess antifungal properties were oximes (2-4). While one phenylhydrazone (XXIX) showed moderate activity against all three organisms, another (III) was only very weakly active against two organisms. All the semicarbazones (IV, XXX), thiosemicarbazones (V, XXVI), and *p*-nitrophenylhydrazones (VI) tested showed little or no activity.

Both of the isonicotinoylhydrazones (VIII, XXVII) were inactive against the fungi. The unsaturated ketone (XIX) formed by the Claisen reaction was somewhat active. The aminoguanidine derivative (IX) was one of the most active compounds in this series. It may be interesting to point out that another antifungal antibiotic, Eulicin, is a guanidino compound (20).

Of the imines, or Schiff's bases, prepared from the various aromatic and heterocyclic amines, the aniline and aminopyridine derivatives (X, XVI) were among the most active agents found in this investigation. Except in the case of compound XXIV, no monosubstituted aniline derivative showed significant activity. Compound XXII, however, with both chlorine and nitro groups on the benzene ring, indicated an enhanced activity.

## REFERENCES

- (1) Noguchi, T., and Okeda, H., *J. Pharm. Soc. Japan*, **76**, 386(1956), *Chem. Abstr.*, **50**, 13746g(1956).
- (2) Doerner, M. P., U. S. patents 2,733,268 and 2,734,031, *Chem. Abstr.*, **50**, 11363d, 15581c(1956).
- (3) Hoffman, M. N., U. S. pat. 2,712,031, *Chem. Abstr.*, **50**, 5748i(1956).
- (4) Ward, W. C., Prytherch, J. P., and Cramer, D. L., *THIS JOURNAL*, **37**, 317(1948).
- (5) Weiner, A. L., and Fixler, Z. C., *J. Am. Med. Assoc.*, **169**, 346(1950).
- (6) "New and Nonofficial Drugs," J. B. Lippincott Co., Philadelphia, Pa., 1959, p. 45.
- (7) Jenkins, G. L., and Hartung, W. H., "The Chemistry of Organic Medicinal Products," 3rd ed., John Wiley & Sons, New York, N. Y., 1949, p. 514.
- (8) Williams, D. I., Marten, R. H., and Sarkany, I., *Lancet*, **2**, 1212(1958).
- (9) Grove, J. F., MacMillan, J., Mulholland, T. P. C., and Royers, M. A. T., *J. Chem. Soc.*, **1952**, 3077.
- (10) Goettsch, R. W., and Wiese, G. A., *THIS JOURNAL*, **47**, 319(1958).
- (11) Bisagni, M., Buu-Hoi, Ng. Ph., and Royer, R. J., *Chem. Soc.*, **1955**, 3688.
- (12) Dey, R. B., and Row, K. K., *ibid.*, **107**, 1651(1915), **123**, 3375(1923).
- (13) Kakimoto, S., Sekikawa, I., and Nishie, J., *Japan J. Tuberc.*, **1**, 42(1953).
- (14) Reichstein, T., *Helv. Chim. Acta*, **13**, 1278(1930), *Chem. Abstr.*, **25**, 1245(1931).
- (15) Tanaka, S., *J. Am. Chem. Soc.*, **73**, 872(1951).
- (16) Shriner, R. L., Fuson, R. C., and Curtin, D. Y., "The Systematic Identification of Organic Compounds," 4th ed., John Wiley & Sons, New York, N. Y., 1958, pp. 131, 218, 219, 254.
- (17) Kosuge, T., *Pharm. Bull. Tokyo*, **2**, 435(1954); *Chem. Abstr.*, **50**, 12187a(1956).
- (18) Huitric, A. C., Pratt, R., Okano, Y., and Kumer, W. D., *Antibiotics & Chemotherapy*, **6**, 290(1956).
- (19) McGowan, J. C., Brian, P. W., and Hemming, H. G., *Ann. Appl. Biol.*, **35**, 25(1948).
- (20) Harman, R. E., Ham, E. A., Bolhofer, W. A., and Brink, N. G., *J. Am. Chem. Soc.*, **80**, 5173(1958).

# Mixing of Pharmaceutical Solids: The General Approach\*

By DAVID TRAIN

IT IS IMPORTANT to have a suitable criterion for good mixing for pharmaceutical solids, because the attainment of an accurate dosage of a small quantity of a potent drug so often depends on the thoroughness by which it is dispersed through the bulk of another, or other materials. Mixing of solids, of necessity, presupposes the particulate form, and there are certain factors and properties associated with particles that will always affect the results of a mixing operation. It is the purpose of this paper to review these factors and properties, and also the operation, so that desirable criteria and possible practical procedures may be assessed rationally when a specific problem in solid mixing is being considered.

Consider an assemblage of particles or a static powder bed before any mixing process begins. All particles are subject to a constant force of gravity and they are in some sort of spatial equilibrium with one another. As long ago as 1885 Osborne Reynolds (1) showed that in order to obtain relative particulate movement within such a bed, the volume of the bed must be increased. Subsequent work by Jenkin (2), and by Brown and Hawksley (3) have amply confirmed that even in the case of a randomly packed bed, no appreciable movement can take place while the bed is in the condition that it spontaneously takes up as it is formed. Brown and Hawksley found that movement in a bed was produced by failure along a slip line or, in other words, a shear movement. For such a movement to take place, there must be sufficient space between the particles and this provides the first necessary condition for mixing. Assuming there is a preliminary condition of partial or total segregation, it follows that mixing must be achieved by some form of interparticulate movement, and this can only take place if the bed of particles is expanded. The extent to which a powder mass may be made to dilate is a complex function of the physical properties of the materials forming the system, but a good empirical test is to compare the specific volume of a tapped bed (4) with the specific volume of the same bed which is on the point

of being fluidized. The ease with which movement could take place is, within reason, a direct function of the degree of expansion which can be produced in the specific volume of the particulate system. Practically, this means that there should be sufficient room in a mixing container to allow the powder mass to dilate, and it also points to the risk of loss of economic efficiency if the space in a given mixer is overfilled.

Assuming interparticulate movement is possible, then, in order to produce such movement, suitable forces must be applied to the particles. Since continued application of pure tensile or compressive forces will serve only to increase or decrease the specific volume of the system without the particles changing stations relative to one another, it follows that shear forces will be necessary to produce interparticulate movement. See Fig. 1. Because the force of gravity is constantly applied, a vertical force either reduces or reinforces the vertical stress according to whether its direction is upwards or downwards. Any horizontal component of force acts at right angles to the gravitational force, and will, in combination with the vertical force, automatically induce a shear stress into that part of the system. If the system is incapable of resisting such a stress, movement along a slip plane takes place.

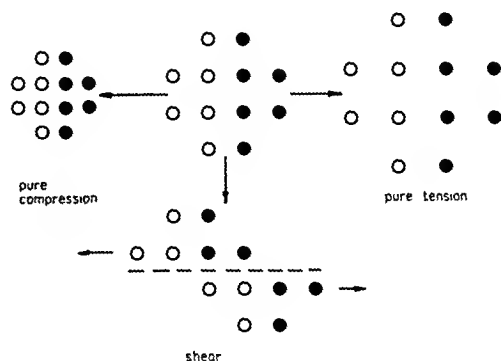


Fig. 1.—Action of forces acting on a particulate system.

The forces are introduced by some external means and, by interparticulate transmission, act within the bed. To ensure mixing there must be such movement that each and every particle could visit, if given time, every point within the con-

\* Received August 21, 1959, from the School of Pharmacy, University of London, England  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August, 1959.

fines of the system in its expanded state. This requires a three-dimensional stress system which will induce movements in all planes. To ensure rapid, and therefore economic, mixing it is necessary to cause the greatest possible number of slips per unit of time. If adjacent slip planes are produced so that they are not more than one particle apart then there will be movement between all particles.

These considerations give, *a priori*, requirements of any apparatus to be used for mixing solid particles. The specific volume of the system must be suitably increased to give freedom of movement to the particles. Forces must act so that a three-dimensional grid would be necessary to plot the lines of movement of each and every particle. These forces must be such that, in time, each particle could visit every point within the confines of the particulate system in its expanded condition. Finally, when all movement ceases the system must be able to take up a state of static equilibrium without segregation of particles taking place. Applied to any specific situation three possible causes of inefficient mixing are highlighted: (a) Insufficient dilation of the bed, either generally or locally, this causes hindrance to particle movement and in its mildest form causes increased time for mixing, and under the worst conditions would prevent any possibility of proper mixing being achieved. (b) Induced forces being insufficient to produce suitable movement in all directions, thus leaving portions of the assemblage undisturbed. This can be manifest as movement in two axes only as in a simple cylindrical drum revolving horizontally about its axis when there is little or no lateral particulate movement. Inadequate forces may also allow the presence of dead zones within a system, or may allow aggregates (loose assemblages of particles held together by light cohesive surface forces such as those caused by water films or electrostatic charges) to move round as compound entities, within which no relative movement or particle exchange takes place. (c) Preferential movement of a certain type of particle due to dissimilarity of physical property (e.g., size or density) of materials being mixed. This can be the cause of imperfect mixing, however long the operation is allowed to proceed, or segregation as soon as mixing conditions change, such as stopping at the end of the process.

#### EFFECTS PRODUCED BY DISSIMILAR PARTICLES AND SURFACE-ACTIVE FORCES

Before considering the criteria for mixing, it is necessary to summarize the effects produced by

certain physical conditions which can occur in the materials to be mixed.

First, there is the difference in size between particles. Coulson and Maitra (5) have reported careful investigations using a simple drum mixer with its axis set at an inclination of  $23^\circ$  to the horizontal because this angle gave best mixing conditions. They found that if fine particles were put at the bottom of the drum and coarse particles of the material placed on top, no mixing occurred. When the position of the sizes was reversed, the larger being on the bottom, then mixing did occur for a short time but segregation developed as the coarse particles rose to the top of the bed. The reason for this difficulty of producing or maintaining a mix with particles of different sizes is due to the small particles being able to fall through the void spaces between the large ones. See Fig. 2. A small particle,  $d = 0.41D$ , can just slip between four larger particles touching in a square grid, while a particle  $d = 0.15D$  can just slip between three larger particles touching on a triangular grid.

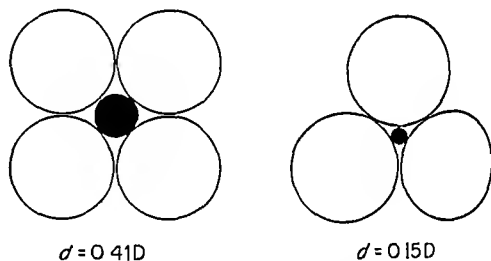


Fig. 2—Small particles slipping between large particles

Practically, it must be recognized that with any mixing apparatus, there is always a tendency for segregation if there are large differences in particle sizes. It is also important to note that even if a true mixture is obtained, the condition is unstable and will separate rapidly if subjected to vibration in storage or subsequent processing. Thus, although it must be accepted that some mixtures have to be made with materials of different sizes, it should always be the aim to mix components with the same size range and the closer the range, the simpler the operation.

A second condition may be caused by a density difference between the component materials. The effect is not so marked as that of difference in size, but Coulson and Maitra showed that the rate of mixing was impeded by an increase in density difference. This was supported by Gray (7) who also found that there was a tendency for segregation to develop with time of mixing in the case of certain systems, like the concrete mixer.

The prevention of segregation in this case requires a condition of vigorous movement between the particles coupled with a restriction on freedom to move in order to prevent segregation under free falling conditions. This is a difficult specification to comply with in practice. Machines which reverse the gravitational field help to reduce the adverse effect of density difference. Again vibration during subsequent processing or storage will help to produce segregation of the mixture.

The effect of particle shape on mixing conditions has not been extensively investigated, but it is well known that roughly equidimensional particles are easier to mix than any other shape. The addition of flat or acicular particles prolongs the mixing time because of their tendency to bundle, and vigorous treatment is often required to break up such assemblages.

The fourth condition is the part played by surface-active forces so that groups of particles are held together as aggregates and consequently do not disperse evenly through the other components. The forces which produce this are mainly surface tension due to adsorbed liquid films, electrostatic charges, or possibly to weak Van der Waals forces. Since the forces act on the surface of the particle, their effect becomes greater as the specific surface area of the solid increases, that is, as the absolute particle size becomes smaller, and produces powders which have high angles of repose and poor flowing characteristics. Andreasen (8) made some investigations into the "stickability" of powders and found that, in those materials which had a propensity to aggregate, the effect became very marked with particles smaller than  $10\ \mu$ . This figure is quite arbitrary but it is a useful one to use because it conveniently provides an upper size limit of what could be subjectively called a "very fine powder." Neumann (4) was interested in setting up an index of "stickiness" and she achieved this by a technique developed by Hawksley (9). Briefly, she found out how much of a coarse granular material she required to add to a given sticky powder in order to make the resultant mixture have certain flow characteristics so that it would maintain a steady flow through a standard orifice. The results for a selection of powders is given in Table 1.

To explain the change in conditions produced in the powder, Andreasen postulated that the fine particles were adsorbed onto the surface of the large particles and as the mass of a large particle was sufficient to overcome aggregation by surface forces, the material became free flowing. As an investigation, Andreasen's experi-

TABLE I.—STICKINESS OF POWDERS

Material	Mean size, $\mu$	Sand Required to Produce Free Flow, %
Catalyst microspheres	25	0
Calcium fluoride	30	0
Anhydrous sodium carbonate	100	23
Titanium dioxide	0.5	26
Refined kaolin	2	1,200
Natural fuller's earth	0.1	25
Activated fuller's earth	5	60
Cement	10	100
Flour	25	30

ments were successful, but the inferences must be applied with care to the mixing of pharmaceutical powders. For example, there is no evidence that all the aggregates of fine powder are broken up when sufficient coarse material has been added to make the mixture flow. Thus, compound particles can still exist and dispersal on a microscale is patchy and irregular even though the random distribution of the large particles through the mix may be perfect. Also, Andreasen was concerned with making a powder with poor flow qualities free flowing; he did very little to investigate the extent of segregation which can be produced with such a mixture under suitable conditions of subsequent processing and storage.

With the foregoing as a basis it is now possible to select a few examples to illustrate some of the inherent limitations in performance of various types of mixing equipment. Reference has already been made to the poor mixing ability of a simple cylindrical drum revolving about its axis because most of the individual particulate movement within is in one plane only. Various mechanical devices have been introduced from time to time to overcome this weakness. The addition of helical flights in one form or another was probably the first modification, and of recent years, revolving the drum with its axis at an angle to the horizontal, or on an axis which is at an angle to an axis of symmetry and to the direction of gravity, has been used successfully on a commercial basis. Into this category, too, may be placed the rotating cube-type mixer. The Z-blade mixer and planet mixer exhibit other variations in that movement is induced by impellers, while the container remains stationary. All techniques induce a skew movement on the particles as they fall under the influence of gravity. This enhances the mixing quality of the system, and is usually successful for mixing free flowing particles of even density. If density differences have to be accommodated then mixers producing a periodic abrupt reversal of flow of the powder, such as the double, V-, or Y-cone

mixers with baffles are the equipment of choice. However, in all the types of plant mentioned above it must be remembered that intensity of shear between particles is mild, and while little or no attrition of other particles may be a desirable property in the case of blending free flowing powders, the break-up of closely held aggregates of small primary particles of a specific ingredient is an inefficient process.

Because size reduction equipment relies almost exclusively on the principle of shear to break down solid material, it is to be expected that most types of plant used for this process have, at one time or another, been utilized for mixing powdered solids. Shear forces in mixing are especially important to help the break-up of the compound aggregates into primary particles. The use of such plant, however, means reducing in size by mechanical breakage at least a proportion of the particles. That this will happen is often anticipated and in such cases ingredients, which are initially oversize, are fed in the correct proportions into the plant and are reduced and partly mixed in one operation. It must be emphasized that although size reduction machinery is used for the mixing process because of the shear forces used, this does not necessarily make such plant good mixing apparatus; there may be efficient incidental mixing on a local scale, but general mixing either does not take place or if it does, proceeds slowly and may therefore be uneconomical.

Muller-type reducers, as exemplified by the pestle and mortar, the end or edge runner mills, or the buhr-stone mill, produce a shearing action between two surfaces moving parallel to the plane of contact. Only a small portion of the material is being processed at any one time and general mixing is slow because the bed is virtually in an unexpanded state and there is little relative movement in the bulk of the particles except near the zone of the muller.

With size reducing plant using impact principles, such as the pin disk mill, the hammer mill, or the fluid energy mill, intense shear forces on a local scale produce a breakdown to primary particles and turbulent air currents ensure mixing of any particles which happen to be in the plant at the time. However, the hold-up capacity of such machines is usually low, so that little or no general mixing is possible and therefore this must be done by other means.

Sieving and sifting techniques have often been recommended as suitable for aiding a mixing process, on the basis that a sieve helps to break up an aggregate or a concentration of an ingredient into primary particles, or, alternatively, the meshes act

as simple proportioning device and redistribute the material as it lands on the sieve. This second premise is probably correct and is a useful procedure when the size of the holes is large compared with the dimensions of the particles. The basis for breaking down aggregates is not so well founded however, because a mesh size must be selected which is only a little larger than the largest particles. Since, on a probability basis, the smaller particles will tend to pass through first it follows that there will always be a segregation on a particle size basis during the process, thus defeating the object of mixing.

### PHARMACEUTICAL REQUIREMENTS OF THE MIXING OPERATION

Having set out the principles of mixing and briefly reviewed the limitations of the apparatus used to achieve it, it is necessary to consider the use to which this operation is put. Pharmaceutical materials are being mixed and therefore by implications, are essentially for medicinal or veterinary use. The physician or veterinarian prefers to use medicaments which produce precise and dependable effects. This can only be achieved by using carefully standardized materials in exact quantities, and the pharmaceutical world has built up a reputation for supplying most of its dosage forms in this manner by suitable process and analytical control. To be successful such control has to be used intelligently, and this applies especially in the case of any dosage forms involving the use of mixtures of powders. Much has been said and written about the procedure for sampling particulate matter when every effort is made to obtain a representative sample of a batch of material. Such routines are correct when a commercial transaction is involved, for it is an assessment of the average content of the ingredients that is required. But when a powder is to be used as the basis for a medicine, then it is the actual content of a specific single dose which must be the criterion for control and sets what has aptly been named "the scale of scrutiny" (6).

The concept may be clearer to understand if one quotes a few examples: the scale of scrutiny for a foot powder or a dusting powder is the amount of an average application; for an internal medicine it is the dose unit or minimum normal dose, a teaspoonful of effervescent granules, the contents of a capsule or cachet, a suppository, or a tablet.

The physician and patient have every right to expect that the stated amount of medicament is, in fact, present in each and every dose product and that the acceptable variations of amount fall

TABLE II.—RELATION BETWEEN MINIMUM UNIT DOSE AND ANALYTICAL SAMPLE OF SELECTED TABLETS IN U. S. P. XV AND B. P. 1958

Ref.	Medicament	Analytical Limits, %	Scale of Scrutiny, Dose, mg.	Assay Sample		Analytical Sample	
				Dose Units Used	Wt. as Active Principle, mg.	Wt. as Active Principle, mg.	Equivalent Dose Units
B. P.	Acetomenaphthone	92 $\frac{1}{2}$ –107 $\frac{1}{2}$	0.5	400	200	200	400.0
B. P.	Ascorbic acid	90–110	25.0	20	500	50	2.0
U. S. P.	Atropine sulfate	90–110	0.3	200	60	60	200.0
B. P.	Carbimazole	90–110	5.0	20	100	6	1.2
U. S. P.	Colechicine	90–110	0.5	50	25	25	50.0
U. S. P.	Cortisone	90–110	25.0	10	250	25	1.0
B. P.	Cortisone	90–110	25.0	20	500	10	0.4
U. S. P.	Digitoxin	90–110	0.1	50	5	2	20.0
U. S. P.	Reserpine	90–110	0.1	20	2	1	10.0
U. S. P.	Stilbestrol	90–110	0.1	100	10	10	100.0

within precise and, preferably stated, overall limits. Mixtures of powders and products derived therefrom may, in fact, fail to comply with this requirement. The final analytical control provides the last check to ensure that the product contains the stated dose of medicament, and it follows from medical requirements that the analytical techniques adopted should, and even must be able to, check adequately the magnitude of minimum unit dose and also provide assessment of the possible variations in amount per unit dose due to limitations in manufacturing procedures.

The ideal condition is to be found in a simple random analytical sample, the size of which is smaller than the minimum unit dose, in which the analyst is confident that his analysis truly represents the specification of the bulk of the material and would be identical with that of any other random sample of the same size. In this case it may be unreservedly assumed that any dose unit contains the required amount of medicament. Such a condition is found in medicaments in the form of simple solutions with low viscosities of say less than 10 centipoises. If, however, the size of the analytical sample exceeds the normal minimum dose, uncertainty of the accuracy of the dose must always exist to a lesser or greater extent according to the number of dose units which are used to make up the analytical sample. This is because there is the possibility of variation in content within the units comprising the sample. From a physician's or patient's point of view, these unknown variations will be in addition to those included in the range allowed by the analysis as well as those inherent in measuring the dose unit concerned. These unknown variations will be small or nonexistent in materials which can be adequately mixed but in the case of materials which are difficult such as powders, they can be quite large so that apparently

identical unit doses could produce important differences in pharmacological response.

To illustrate this point reference should be made to the official requirements for some products of powders, i. e., tablets, in the U. S. P. XV and B. P. 1958. (See Table II.) The smallest dose in which the medicament is normally prescribed has been selected because it is in this condition that, between doses, the greatest variation is possible. It will be seen that 400 acetomenaphthone B. P. dose units or 200 atropine sulfate U. S. P. dose units are required to produce a minimum analytical sample. Thus the variations to be found between 200 individual doses of atropine sulfate have been averaged to give a mean content of drug with an allowable error of 10% about the stated content. Thus any imperfection of mix in any one tablet will be masked by the contribution made by 199 units which also form the analytical sample. On the other hand only two-fifths of the minimum dose of cortisone is required as the analytical sample for the B. P. assay yet the analyst has been required to take 20 such dose units to make his sample. Other examples speak for themselves. It will be seen that the emphasis has been to obtain a reasonable mean and is probably favorable to the manufacturer. Products only fail to pass the test if there has been a gross error of judgment in manufacture when, correctly, the batch should be rejected.

It has been deemed necessary to check the uniformity of weight of individual products, but no method is given or suggested so that the uniformity of mix may be checked on a minimum dose unit scale. Thus the uniformity of mix of the ingredients is left to the inherent mixing capability of the machine and the integrity of the operator to run the machine to give the optimal conditions of mix if he knows them.

In practice, where the pharmacological intention of the dose is to correct a deficiency or



maintain a medication, as in ascorbic acid or digitoxin, the patient's body probably can accommodate the possible product-to-product variation in dose, but when the analytical sample rises above 50 dose units, the course of treatment must be long before it can be safely assumed the patient has taken a mean dose consistent with the mean content as shown by analysis. When medication depends on the effect produced by only one or two dose units, then it is reasonable to assume that sufficient attention is paid to the checking of individual dose units. It should be a matter of professional pride that this is done. However there are certain principles of probability which come into the process of mixing and these must be clearly borne in mind when a mixing procedure or checking scheme is being developed.

Because there is a trend towards dry mixing of powders for slugging in tablet manufacture and for filling capsules, it is a useful exercise to assess the statistical aspects of obtaining a unit dose with the correct proportion of ingredients from a truly randomized bulk mixture of equal sized particles of ingredients present in their correct proportions. The probability of obtaining a sample containing the correct proportions of the ingredients varies with the number of particles taken for the sample (10). Thus, in taking samples from an infinite supply of a 50:50 mixture of *P* and *Q*, a sample of *n* particles will on an average contain *n*/2 particles of *P*. But any one sample may contain less than this number with a corresponding increase in *Q*. The error to be expected can be expressed mathematically in the form given by the standard deviation about the mean, and it can be shown that multiples of the standard deviation may be used to determine confidence limits to give the proportion of samples, consisting of a given number of particles, that would have less than a given variation in the proportion of a selected ingredient.

For example (See Table III), in the case of a 50:50 mixture, the standard deviation of one component, expressed as a percentage of the whole, is given by  $100/\sqrt{n}$ , where *n* is the number of particles in the sample. This means that if

TABLE III.—PERFECT MIXING<sup>a</sup>

Confidence Level of No. of Samples	% Limiting Error in <i>p</i>		
	<i>n</i> = 100	<i>n</i> = 10,000	<i>n</i> = 1,000,000
68.3	10	1	0.1
95.5	20	2	0.2
99.7	30	3	0.3

<sup>a</sup> Proportion of *P*:*Q* = 50:50; no. of particles in a sample *n*; standard deviation of sample, % =  $100/\sqrt{n}$ .

a sample of 100 particles were taken, one could be confident that 68.3 per cent of samples would have less than a 10 per cent error in the proportion of *P*, and in like manner 95.5 per cent of samples would have less than a 20 per cent error, while 99.7 per cent samples would have less than a 30 per cent error in *P*. If the sample size *n*, were raised to 10,000 particles the errors for each confidence level will have reduced to 1, 2, and 3 per cent, respectively, while if *n* is a million particles, the errors will have been reduced to 0.1, 0.2, and 0.3 per cent of the desired proportion.

If, however, the proportion of one ingredient is small (see Table IV), then the standard deviation

TABLE IV.—PERFECT MIXING<sup>a</sup>

Confidence Level of No. of Samples, %	% Limiting Error in <i>p</i>		
	<i>p</i> 0.01 <i>n</i> 900	0.01 250,000	0.03 1,000,000
68.3	30	2	2
95.5	60	4	4
99.7	90	6	6

<sup>a</sup> Proportion of *P*:*Q* = *p*:1-*p*; no in sample = *n*; standard deviation of sample, % =  $100/\sqrt{pn}$ .

from the mean of the proportion of a given ingredient, expressed as a percentage, may be estimated by  $100/\sqrt{pn}$ , where *p* is the proportion of the material expressed as a fraction and *n* is the number of particles in a sample. It will be seen that for a 1 per cent dispersion and a sample of 900 particles, the confidence levels will be as follows: 68.3 per cent samples would have less than 30 per cent error in the proportions of *P*, 95.5 per cent samples would have less than 60 per cent error in the proportions of *P*, and 99.7 per cent samples would have less than 90 per cent error in the proportions of *P*. To assume reasonable errors of 2, 4, and 6 per cent, respectively, the total number of particles per sample must be of the order of 250,000. Applied to dry mixing of powders, this means that for a dose of 1 mg. of drug in a product weighing 100 mg. the total material should be reduced in size to give at least 250,000 particles so that the unit dose, based on a perfect mixing operation alone, may be within 4 per cent of the correct dose for 95.5 per cent of the products and within 6 per cent for 99.7 per cent of the products. Assuming that the drug and diluent have a specific gravity of 1.5, the mean particle size of all ingredients must be less than 50  $\mu$ .

One of the smallest doses in either pharmacopia is 0.1 mg. Assuming that this is to be presented as a 40-mg. dose unit, the dilution is 1 in 400. Working to the same confidence

limits as the preceding example, the unit sample should contain at least 1 million particles so that the mean particle size should be less than  $\frac{1}{2}\mu$ .

It must be emphasized that the foregoing calculations have been based on a condition of perfect mixing. If this is not obtained, the variation in behavior between unit doses is subject to additional errors. It is clear that the operation of powder processing is by no means understood or worked out and that the worst enemy in this field is probably complacency through ignorance. Pharmaceutically it must always be the aim to supply precise individual dose units, but it

must be concluded that present day practices in powder technology sometimes fall short of this attainment.

## REFERENCES

- (1) Reynolds, O., *Phil. Mag.*, 20, 469(1885).
- (2) Jenkin, C. F., *Proc. Roy. Soc., A*, 131, 53(1931).
- (3) Brown, R. L., and Hawksley, P. G. W., *Fuel*, 26, 159 (1947).
- (4) Neumann, B. S., "Flow Properties of Disperse Systems," Interscience Publishers, Inc., New York, N. Y., 1953.
- (5) Coulson, J. M., and Maitra, N. K., *J. Imp. Coll. Chem. Eng. Soc.*, 4, 135(1948).
- (6) Anon., *Brit. Chem. Eng.*, 1, 29(1956).
- (7) Gray, J. B., *Chem. Eng. Progr.*, 53, 25(1957).
- (8) Andreasen, A. H. M., Hofmanbank, N., and Rasmussen, N. H., *Kolloid-Z.*, 86, 70(1939).
- (9) Hawksley, P. G. W., *Pulverized Coal Conference*, 1947, 679.
- (10) Saunders, L., and Fleming, R., "Mathematics and Statistics," Pharmaceutical Press, London, England, 1957.

## Studies on *Morinda citrifolia* L. II\*

By HEBER W. YOUNGKEN, Sr., HOWARD J. JENKINS, and CALVIN L. BUTLER†

The results of a study of the anatomy of the stem of *Morinda citrifolia* L. are described. A preliminary report of the pharmacodynamic study of a 95 per cent alcoholic extract of the root on the isolated uterine horn of an albino rat showed some measure of sedation using doses of 1 to 5,000 to 1 to 36,000. A water decoction of the crude whole root, injected intravenously in the anesthetized dog, in a dose representing 4 Gm. produced a marked lowering of blood pressure which lasted approximately twenty-six minutes. It appears that the hypotensor activity resides in the water-soluble fraction of the root of *Morinda citrifolia*. The results of *in vivo* experiments gave evidence of a low order of toxicity of *Morinda citrifolia* root.

AN INVESTIGATION of the root of *Morinda citrifolia* L. and of herbarium material of the same species was reported in a previous paper (1). The studies reported here include an investigation of the anatomy of the stem of *M. citrifolia*. *In vitro* tests on segments of the anterior horn of the albino rat uterus were carried out with an alcoholic extract of the root, and the effects of intravenously injected alcoholic and aqueous extracts of the root were observed for hypotensor action in rabbits and dogs.

### PREPARATION OF MATERIALS‡

The materials were prepared for anatomical studies by repeatedly boiling and sectioning stem segments approximately 5, 12, and 18 mm. in diameter. The bark portions were placed between the

flat surfaces of cork prior to sectioning by the sliding microtome and by free hand. Transverse, radial longitudinal, and tangential longitudinal sections were cut and mounted separately in water, dilute glycerin, 2% potassium hydroxide solution, iodine water, and phloroglucin-HCl. Schulze's maceration method was employed to facilitate the separation of the lignified from the nonlignified elements, followed by teasing them apart on the microslide and mounting for microscopic study. Structures were studied under the low and high magnifications of the microscope. Scrapings of representative parts of the branch were also examined microscopically in these reagents and the results recorded. Starch grains were examined and measured in water mounts of scrapings and powdered stem material.

### ANATOMY OF THE STEM OF *Morinda citrifolia* L.

Examination of transverse sections 5 mm. in diameter revealed the following structure: (a) an epidermis of clear cells with strongly cutinized outer and radial walls; (b) a hypodermis of two zones, the outer of which was composed of a layer of parenchyma, the inner zone consisting of two layers, the outer of an almost continuous band of stone cells interspersed here and there with groups of sclerenchyma fibers, the inner layer

\* Received August 21, 1959, from the Massachusetts College of Pharmacy, Boston.

† Chief Chemist and Director, Essex Research Foundation, Lynn, Mass.

‡ Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

§ The materials used in these studies consisted of an entire long branch of *M. citrifolia* collected from a plant growing in the Indian Museum Garden, Calcutta, India, by Dr. Sunil C. Datta; and about 20 pounds of roots and 20 pounds of stems of *M. citrifolia* gathered in India by collectors of the S. B. Penick Co.

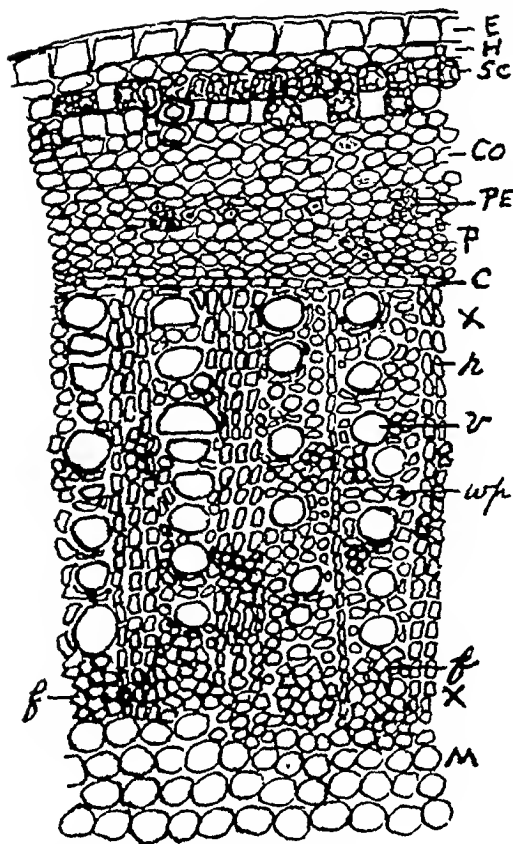


Fig. 1—Representative portion of a transverse section of a distal segment of a stem 5 mm in diameter of *Morinda citrifolia* L. E, epidermis, H, hypodermis containing stone cells and groups of fibers (Sc); Co, cortex, Pe, pericycle, P, phloem, C, cambium; X, xylem; r, xylem ray; v, vessel, wp, wood parenchyma, f, wood fiber, M, pith. Magnified.

consisting of parenchyma and scattered stone cells with an occasional small group of sclerenchyma fibers; (c) a cortex consisting of four layers of cortical parenchyma some of the cells of which contained small starch grains; (d) a pericycle of about three layers and consisting chiefly of ordinary parenchyma in which groups of pericyclic fibers were scattered; (e) a narrow phloem of sieve elements and phloem parenchyma traversed by phloem rays, (f) a wavy cambium of meristematic cells; (g) a broad xylem of numerous wood wedges separated from each other by xylem rays from 1 to 3 cells wide. The walls of the latter were pitted, non-lignified to lignified. The wood wedges contain vessels which are mostly isolated and also arranged radially, numerous tracheids and groups of wood fibers as well as considerable apotracheal wood parenchyma with pitted walls. Prominent groups of wood fibers with strongly lignified walls appeared especially abundant near the inner margin of the wedges. The xylem rays were composed mostly of radially elongated cells with nonlignified to lignified walls. They were interrupted in places by groups of fibers with lignified walls; (h) a pith composed of rounded pith parenchyma with cellulose walls

which showed pitting. This region already showed evidence of centrifugal disintegration as was shown by its hollow center. See Fig. 1.

Longitudinal radial sections showed axially arranged raphide saes containing acicular crystals of calcium oxalate up to 100  $\mu$  in length, occurring in both cortex and phloem, spiral, simple pitted, and bordered pitted vessels and tracheids with lignified walls, elongated wood parenchyma cells with lignified pitted walls, and thick- and thin-walled wood fibers with oblique pits in their lignified walls.

Transverse sections of the stem from 8 to 18 mm in diameter were characterized by a progressive increase in the number of sclerenchyma elements in the hypodermis and pericycle, by the occurrence of scattered groups of fibers in the phloem, an increased lignification of the walls of the xylem elements, and an increased disintegration of the pith in centrifugal fashion. See Fig. 2.

The vessels were isolated and in small groups of 2 to 4 and in radial groups of 2 to 10. In longitudinal sections of the same stem diameters, their ends were oblique, straight, or rounded and exhibited large circular openings in their perforation plates. They measured up to 684  $\mu$  in length and up to 114  $\mu$  in width.

Longitudinal radial and tangential sections of the stem, 8 to 18 mm. in diameter, showed a progressive decrease in the number of raphide saes and an

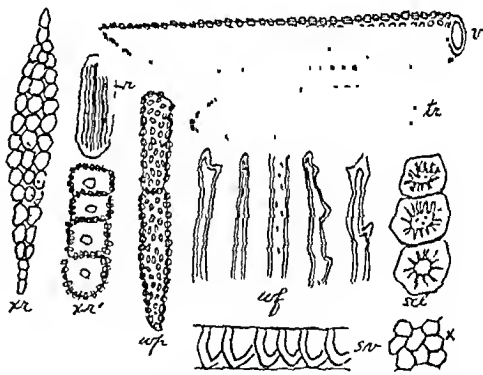


Fig. 2—Diagnosical histological elements occurring in the stem of *Morinda citrifolia* L. Xr, xylem ray as observed in a tangential section, tr, 4 cells of a xylem ray as observed in a transverse section, r, raphide sac containing acicular crystals of calcium oxalate, wp, wood parenchyma, v, pitted vessel; tr, pitted tracheid, sr, spiral vessel, wf, wood fibers, scl, stone cells; K, cork.

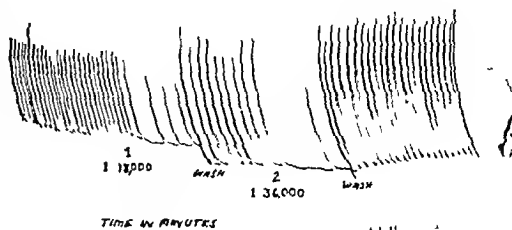


Fig. 3—Effect of *Morinda citrifolia* root 95% alcohol Soxhlet extract on the right horn, isolated uterus of an anestrous rat, in vitro.

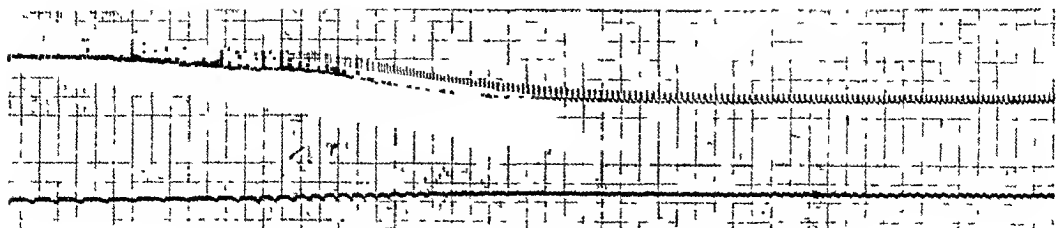


Fig 4—Studies on *Morinda citrifolia*, effect on blood pressure

increase in the number of stone cells in the bark. The tracheids were of the same types as the vessels but mostly shorter and always with closed ends. The wood fibers were straight to curved, a number showing lobing or dentation toward one or both ends. Their ends were mostly acute or attenuate, their walls strongly to moderately lignified with oblique pits, and their lumina irregular in width. They measured up to 1,400  $\mu$  in length. A number of the parenchyma cells possessed globules of oil. The starch grains were fewer than those previously observed in the root, and mostly single. They measured up to 15.2  $\mu$  in diameter.

PRELIMINARY *IN VITRO*  
PHARMACODYNAMIC STUDY

Preliminary *in vitro* pharmacodynamic experiments upon isolated tissues were made in accordance with the Magnus method as described by Burn (2). Segments of the isolated uterine horn of the albino rat were employed as the test tissue, and used by preference since results were more easily producible. Further, the various stages of the estrus cycle can be easily determined by examination of the vaginal smear before sacrifice. In our hands it was found that the uterine tissue of rats at anestrus responds much more uniformly than at other times of the cycle.

The apparatus used was of the general type and consisted of a constant temperature bath automatically maintained at 37.5°, containing a 100-cc muscle chamber in which a horn or a strip of the uterus, freed from its mesenteric adhesences, was immersed. Oxygenation of the tissue was assured by using a tank of oxygen equipped with a valve which allowed a regular and constant flow of the gas to bubble through the nutrient solution. The nutrient solution was of the following formula:

NaCl (reagent)	9.0 Gm
KCl (reagent)	0.42 Gm
CaCl <sub>2</sub> (reagent) (anhydrous)	0.24 Gm
NaHCO <sub>3</sub> (reagent)	0.5 Gm
Dextrose	0.5 Gm
Distilled H <sub>2</sub> O	q s 1000.0 ml.

It was found that the addition or omission of 2.5 mg. magnesium chloride to this formula makes little difference in the response of the isolated organ and, accordingly, it was omitted in this series of experiments.

A series of ten experiments using a 95% alcoholic extract of *Morinda citrifolia* root was made. It was not found necessary to de-alcoholize the test substance since the small volumes of alcohol involved had been shown previously to have no effect on uterine tissue.

Figure 3 illustrates the typical response we observed in the majority of the experiments.

At (I) a dilution of 1:18,000 of a 95% alcoholic extract was injected into the muscle chamber. A characteristic decrease in activity as witnessed by a decrease in amplitude and rate of contractions with some lowering of tone was shown. Upon washing several times with fresh nutrient solution normal activity was resumed.

At (II) a dilution of 1:36,000 showed still more sedation than the first dose which indicates that the tissue must have been somewhat conditioned to the drug. Normal activity occurred upon washing.

In all the experiments some measure of sedation was shown, using doses ranging from 1 to 5,000 to 1 to 36,000.

THE PRESSOR ACTIVITY OF SELECTED  
FRACTIONS OF THE POWDERED WHOLE  
ROOT OF *Morinda citrifolia*

**Alcohol-Soluble Portion.**—Twenty grams of *Morinda citrifolia* root was exhausted with 250 ml. chloroform in a Soxhlet apparatus for ten hours. The marc was extracted with 250 ml. of 70% ethanol. The ethanol extract was dried under reduced pressure and the orange-red residue was redissolved in 60% propylene glycol. This preparation, injected intravenously into the rabbit in a dose representing 400 mg. of the crude root, failed to bring about a significant lowering of blood pressure.

**Water-Soluble Portion.**—A water decoction of the crude whole root was made by boiling the drug for ten minutes. The decoction was then strained and filtered. The cloudy filtrate obtained was not filtered further. This preparation injected intravenously in the anesthetized dog in a dose representing 4 Gm. of the crude whole root produced a marked lowering of blood pressure which lasted approximately twenty-six minutes, see Fig. 4. This pharmacologic test supports Dr. Van Ho's clinical observations on the hypotensor action of the drug (3).

Time, min	Blood Pressure	Heart Rate
pre-drug	165/95	150
1	135/80	222
2	20/0	108
3	40/15	108
5	110/70	102
9.5	160/105	84
23.5	150/100	114
26	165/95	130

REFERENCES

(1) Youngken, H. W., Sr., *This Journal*, 48, 162 (1958).  
(2) Burn, J. H., "Biological Standardization," Oxford University Press, Oxford, England, 1937.  
(3) Van Ho, D., *Presse méd.*, 63, 1478 (1955).

# Changes Induced by Gibberellic Acid on Growth and Alkaloid Patterns in *Datura stramonium* Linné and in *Atropa belladonna* Linné\*

By ROBERT E. BRUMMETT and LEO A. SCIUCHETTI

Stramonium and belladonna were administered single 100-mcg. doses of gibberellic acid by application to the uppermost leaves of the plants. Growth and alkaloid patterns following treatment are described. A modified assay procedure for 25-mg. samples of material is outlined. The total plant alkaloids in treated stramonium were slightly less than controls at each harvest period. Total root alkaloid production, however, was increased about 50 per cent. Interesting differences in fresh and dry weights were induced in belladonna at different stages of maturity. Younger seedlings indicated significantly decreased growth, whereas older plants demonstrated significant increases. Due to increased growth the older plants displayed about a 32 per cent increase in total alkaloid production. The age of the plant when treated appeared to be an important factor in causing increased alkaloid production.

PREVIOUS RESEARCH (1) has demonstrated that gibberellic acid (G. A.) when administered in the form of an aqueous spray in concentrations of 100 and 1,000 p. p. m. to the leaves and tops of *Datura stramonium* and *Atropa belladonna* produced a favorable effect on the growth of the former, but a less desirable response on the latter. Decreased concentrations of alkaloids were generally noted in the morphological parts of treated plants. Similar trends have been reported in *Hyoscyamus niger* (2) and in different *Nicotiana* species (3).

This study differed from our previous work (1) in that a single 100-mcg. dose of G. A. was administered to the uppermost leaves of the plants, patterns of growth and alkaloid formation were determined from periodic harvests of plant parts rather than from a terminal harvest, and, with belladonna, it was desired to determine whether the age of the plant (more appropriately, the stage of plant development when treated) would be an important factor in the response to the gibberellin effect.

## EXPERIMENTAL

**Procedure.**—*Datura stramonium* plants employed in this study were grown under greenhouse conditions in a previously fertilized<sup>1</sup> plot of ground. Seeds were germinated in culture flats containing soil composed of two parts each of sand and sandy

loam, and one part of peat moss. On June 24, 1958, 56 twenty-two-day-old plants were transplanted into the greenhouse soil plot. These were planted in three rows which were two feet apart and the plants were spaced two feet apart in each row. The plants were then divided into seven groups of eight each and designated as controls and plants to be treated. On July 1, 1958 (zero-time), the 24 plants that had been designated for treatment were given a single dose of 100 mcg. of G. A.<sup>2</sup> This quantity was delivered from a micropipet onto the surface of the youngest unfolding leaf by applying 0.02-ml. portions of a freshly prepared aqueous solution (containing 5 mg. of G. A. per ml.). Height measurements were taken twice weekly. The plan for harvesting stramonium was as follows: 32 control plants were divided into groups of eight each and each group was harvested at zero, one, two, and three weeks; 24 treated plants were similarly divided and harvested at one-, two-, and three-week intervals. Division of the plant into its morphological parts, fresh and dry weight determinations, pulverization, and storage of the powdered material were conducted in a manner described in a previous publication (1).

The belladonna seeds were germinated in a manner similar to that for stramonium. The average germination date for the first planting was April 17, 1958. This planting is hereafter designated as the older plants. The average germination date for the second planting was June 1 (hereafter designated as the younger plants). On June 26, 1958, 80 plants consisting of 40 seedlings from each planting were transplanted into a fertilized soil plot in the greenhouse. Treatment time was July 3, 1958. The younger plants (A series) were thirty-three-day-old seedlings at the start of the experiment; the older plants (B series) were eleven-week-old seedlings. The control group within each series consisted of 24 plants, from which eight each were selected at random and harvested at zero, three, and six weeks. The treated group consisted of 16 plants from which eight each were harvested at three and six weeks. At zero time the treated plants

\* Received August 21, 1959, from Oregon State College, School of Pharmacy, Corvallis.  
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

The work with stramonium was done by Robert E. Brummett and the manuscript resulting from this research was selected as the 1959 Edwin Leigh Newcomb Award.

The study with belladonna was done by Leo A. Sciuchetti and was supported in part by a grant from the General Research Fund of the Graduate School of Oregon State College.

<sup>1</sup> Each plant was furnished with 100 Gm. of organic fertilizer (analysis—5% total nitrogen, 3% available phosphate, 2% available potash) that had been worked into the soil prior to planting.

<sup>2</sup> The gibberellic acid used in this study was furnished through the courtesy of Dr. Edwin F. Alder, Agricultural Research Center, Eli Lilly and Co., Greenfield, Ind.

TABLE I.—WEIGHTS OF BELLADONNA PLANT PARTS FOLLOWING A SINGLE TREATMENT WITH GIBBERELIC ACID (AV./PLANT/GROUP)

Sample and Harvest Time, wk	Total Weight			Leaves-Tops			Stems			Roots		
	Fresh, Gm	Dry, Gm	Control Dry Wt, %	Fresh, Gm.	Dry, Gm	Control Dry Wt, %	Fresh, Gm	Dry, Gm.	Control Dry Wt, %	Fresh, Gm	Dry, Gm	Control Dry Wt, %
<b>A Series</b>												
Control, 0	0 130	0 012		0 100	0 009		a	a	..	0 030	0 003	...
Control, 3	3 64	0 304		3 31	0 25		a	a	..	0 33	0 054	
Treated, 3	2 61	0 248	82	2 69	0 21	84	a	a	a	0 22	0 038	70
Control, 6	44 93	4 59		35 67	3 74	..	5 32	0 38		3 94	0 47	
Treated, 6	26 26	3 18	69	19 47	2 41	64	3 91	0 40	105	2 88	0 37	79
<b>B Series</b>												
Control, 0	0 486	0 032		0 450	0 047		a	a	..	0 036	0 005	..
Control, 3	9 65	0 821		8 29	0 670		a	a	..	1 36	0 151	
Treated, 3	13 11	1 25	152	11 34	1 03	157	a	a	a	1 77	0 199	133
Control, 6	111 35	11 07		82 03	8 51		17 66	1 42		11 66	1 14	
Treated, 6	169 34	16 30	148	112 52	11 24	132	38 08	2 92	206	18 74	2 14	188

a No differentiation into stem portions so shoots were designated as leaves tops A series plants were thirty-three-day-old seedlings at 0 time, B series, eleven week-old plants

were administered 100 mcg of G A in the manner previously described Height measurements were taken twice weekly At zero time and three weeks each plant was divided into shoot (labeled leaves-tops) and root portions At this stage of development stem growth was almost negligible and the small amount available was combined with the leaves and tops By the sixth week, however, stem development was satisfactory so that this additional portion was collected Otherwise, the harvesting procedure was identical with that described for stramonium

**Growth Effects.**—The treated plants generally demonstrated the gibberellin effects previously reported by Smith and Sciuchetti (1) The proneness of treated plants to insect attacks was not noted in stramonium and only to a slight extent in belladonna This may have been due to the fact that the G A was placed on one leaf only in this experiment instead of being sprayed onto the aerial parts of the plant, the soil, and the plant container The insects may have been attracted to the G A itself rather than by any reduced resistance in the plant.

The height increases in both plants were not as great as that induced by a spray treatment (1) With belladonna the maximum increases were attained at the fourth week, an 80% increase was noted in the younger plants and a 106% increase in the older plants The rapidity of response and the sensitivity to this growth effect were more pronounced and of longer duration in the older plants. Bukovac and Wittwer (4) have also reported that the age of bean plants influenced their response

**Fresh and Dry Weights.**—A favorable response on growth was generally induced by the G A treatment in stramonium as indicated by fresh and dry weight data. The growth rate was more rapid than that previously reported (1) Further, the roots demonstrated a beneficial effect (up to an 80% increase in root dry weight at the second harvest) contrasted with the decreased growth reported in earlier work (1). Two factors may have contributed to increased root growth: the plants in this experiment were grown directly in the soil

instead of pots, thus, "root-bounding" was not a possibility; and the plants received fertilizer. Other workers (5, 6) have indicated that fertilization is an important factor in order to induce a favorable growth response from treatment with G. A

With belladonna the younger plants (A series) demonstrated generally significant reductions in fresh and dry weights, whereas the older plants (B series) indicated significantly increased weights (Table I) The age of the plant (stage of plant development) appeared to be an important factor in obtaining a beneficial response from the treatment.

**Analysis for Alkaloids.**—The dried plant parts, using pooled samples, were assayed for total alkaloids by a new procedure which was found to be efficient and accurate for 25-mg. samples of material The method employed the citric acid buffer described by Witt and Youngken (7) for the extraction of the powdered drug and an extractor as described by French and Gibson (8) The extraction procedure was as follows: A small cotton plug was placed in the bottom of the extractor. The 25-mg sample was then placed in the extractor and shaken into a compact column. Another plug was placed gently on the top Citric acid buffer (0.4 ml) was placed on the plant material and allowed to macerate for two hours. The aqueous solution containing the extractive was withdrawn directly into a 25-ml separatory funnel containing 15 ml of chloroform First, a 3-ml. portion of citric acid buffer, and then, two 1-ml. portions of buffer were used to complete the extraction and rinse the extractor. The combined collection of the aqueous phases was made basic to litmus paper by using 28% ammonium hydroxide. The contents of the separatory funnel was then shaken by hand for one minute and allowed to stand overnight. The 15-ml. portion of chloroform was then removed and collected in airtight glass containers. The remaining phase was shaken for one minute, using an additional 10-ml. quantity of chloroform This was then added to the first 15-ml. portion. Ten-milliliter aliquots of the combined 25-ml sample were then analyzed for total alkaloids by the Vitali-Morin reaction.

Significant decreases in the concentration of alkaloids (calculated as scopolamine) were induced by the treatment in the leaves-tops and stems of stramonium while slight changes were observed in the roots (Fig 1). The largest decreases per plant part which were noted were a 30% reduction in the leaves-tops at the third week and a 55% reduction in the stems at the second week

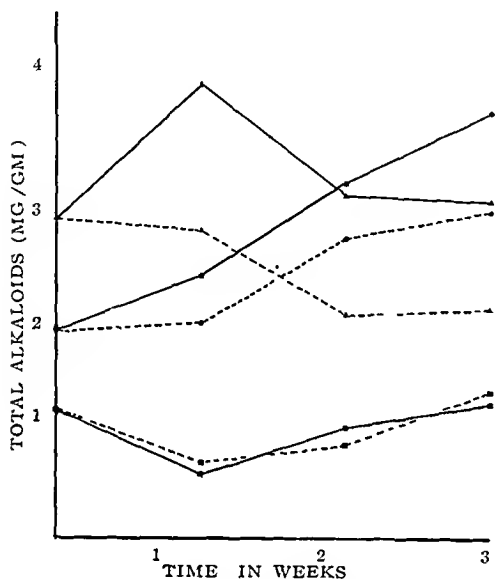


Fig 1.—Alkaloid pattern in plant parts of stramonium, expressed as scopolamine. ●—●, Control leaves; ●·····●, treated leaves, ▲—▲, control stems; ▲·····▲, treated stems, ■—■, control roots, ■·····■, treated roots

The G. A induced a similar effect in belladonna since reduced concentrations of alkaloids (expressed as hyoscyamine) were noted in the parts of the treated plants of each series (Fig 2). The decreases were greater in the younger plants than in the older plants. Decreased alkaloid concentrations resulting from a G. A treatment have also been reported by others (1-3) in members of the solanaceae.

**Total Plant Alkaloids.**—The total alkaloids per plant and per plant organ were obtained by multiplying the dry weight of the plant organ by the per cent of alkaloids obtained from the alkaloid analyses and expressing the results in milligrams (Table II). Slight decreases in the total alkaloid content per plant were induced in stramonium by the treatments. The roots of treated plants, however, demonstrated increases of 40% and 54% for the second and third harvests, respectively. This was due mainly to a significant increase in root growth.

The treatments resulted in diverse effects on the total alkaloid content of belladonna. The younger plants (A series) produced about 40% less alkaloids at the sixth week compared with controls, whereas the older plants (B series) displayed about a 32% increase in the total alkaloid content (Table II). The significantly increased alkaloid production in

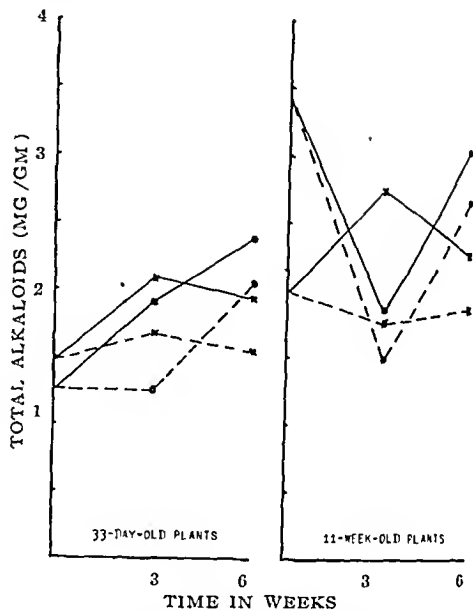


Fig 2.—Alkaloid pattern in belladonna organs, expressed as hyoscyamine. ●, Shoots, ▲, roots, —, controls; ---, treated.

TABLE II—TOTAL ALKALOID CONTENT OF STRAMONIUM<sup>a</sup> AND BELLADONNA<sup>b</sup>

Sample and Harvest Time, wk	Total Alkaloid Content, mg—			
	Per Plant	Leaves and Tops	Stems	Roots
<b>Stramonium</b>				
Control, 0	0.06	0.04	0.02	0.01
Control, 1	0.36	0.25	0.11	0.03
Treated, 1	0.31	0.20	0.08	0.03
Control, 2	2.06	1.71	0.30	0.05
Treated, 2	1.98	1.58	0.33	0.07
Control, 3	12.94	11.17	1.42	0.35
Treated, 3	11.15	8.68	1.93	0.54
<b>Belladonna</b>				
<b>A Series</b>				
Control, 0	0.015	0.011		0.004
Control, 3	0.590	0.478		0.112
Treated, 3	0.324	0.260		0.064
Control, 6	10.70	8.49	1.36	0.851
Treated, 6	6.31	4.94	0.80	0.574
<b>B Series</b>				
Control, 0	0.170	0.160		0.010
Control, 3	1.67	1.25		0.415
Treated, 3	1.94	1.58		0.355
Control, 6	32.74	25.16	4.98	2.60
Treated, 6	43.23	31.47	7.74	4.02

<sup>a</sup> Total alkaloids expressed as scopolamine

<sup>b</sup> Total alkaloids calculated as hyoscyamine

<sup>c</sup> Alkaloid content for plant parts calculated from dry weight and alkaloid analyses data, total plant alkaloid = leaves and tops + stems and roots, based on av./plant/group

the older plants was due primarily to the favorable effect of G. A. in promoting more extensive growth. The age of the plant when treated appeared to be an important factor in causing increased growth which, in turn, resulted in increased alkaloid production per plant.

## SUMMARY

1 A modified assay procedure for the estimation of total alkaloids in 25-mg samples of powdered drug is outlined

2 Characteristic gibberellin effects were induced in stramonium and belladonna by the treatments. In the latter, height measurements indicated that older plants reacted more rapidly and for a longer period of time to this response than did younger plants

3 Slight changes in the total dry weight of stramonium were induced, but root growth was increased up to 80 per cent. In belladonna the total dry weight of the younger plants was reduced about 30% while that of the older plants was increased about 48%

4 The concentration of alkaloids in the organs of each species was generally reduced. Total alkaloid production in treated stramonium

was somewhat less than that in controls, but total root alkaloids were increased about 50 per cent.

5. At the sixth week following treatment younger belladonna plants produced about 40 per cent less total alkaloids than controls, whereas older plants displayed about a 30 per cent increase compared with controls.

## REFERENCES

- (1) Smith, G. M., and Sciuchetti, L. A., *THIS JOURNAL*, 48, 63(1959)
- (2) Masuda, J. V., and Hamur, G. H., *ibid.*, 48, 361(1959)
- (3) Burk, L. G., and Tso, T. C., *Nature*, 181, 1672(1958)
- (4) Bukovac, M. J., and Wittwer, S. H., *Mich. State Univ. Agr. Expt. Sta. Quart. Bull.*, 39, 307(1956)
- (5) Brian, P. W., Elson, G. S., Hemming, H. G., and Radley, M., *J. Sci. Food Agr.*, 5, 602(1954)
- (6) Mitsui, N., Mitsui, T., Hirano, T., and Sarurai, M., "Proceedings 1st Japanese Gibberellin Symposium," Tokyo, 1957, p. 46
- (7) Witt, J. A., Jirawongse, V., and Youngken, H. W., Jr., *THIS JOURNAL*, 42, 63(1953)
- (8) French, O. I., and Gibson, M. R., *Anal. Chem.*, 29, 1166(1957)

## Digitalis X. The Infrared Absorption Spectra of Some Digitalis Glycosides and Aglycones\*

By FREDERICK K. BELL

The infrared absorption spectra of commercial samples of the three lanatosides, their aglycones and their three intermediate glycosides, digitoxin, gitoxin, and digoxin, have been scanned between  $2.5\mu$  and  $15\mu$  using the potassium bromide disk technique. Some of the more pronounced characteristics of these spectra have been noted and their possible significance in the identification and differentiation of these highly important cardiotonic substances has been indicated.

WITH THE RAPIDLY increasing availability of the technique of infrared absorption spectrometry it seems timely that an examination of the infrared absorption spectra of some of the more common digitalis principles should be undertaken. The results of such a study could be reasonably expected to provide useful information for those interested in the isolation, purification, and identification of these highly important glycosides. It also seems probable that because of its unusual scope, the infrared absorption spectrum will assume as increasingly important position along with other well-known physical constants in purity and identification rubrics. In the extensive studies of the infrared absorption of

steroids (1) only one of these digitalis substances, digitoxigenin, and several derivatives have been reported.

For this preliminary study the three lanatosides and some of their degradation products have been selected for examination. This selection was based on several considerations. Commercial availability and also the inclusion of some of the most important cardiotonic agents in clinical use were of prime importance, as well as the interrelationships of the chemical structures of the various compounds.

## EXPERIMENTAL

The substances examined together with the source of supply are as follows: lanatosides A and B (Sandoz Pharmaceuticals), lanatoside C (N. F. Reference Standard), digitoxin (U. S. P. Ref.

\* Received August 1, 1959, from the School of Medicine, University of Maryland, Baltimore 1, Md.  
The author gratefully acknowledges the kind interest and suggestions of Dr. Leopold May of the Psychiatric Institute



erence Standard), gitoxin (Hoffmann-LaRoche and Co.), digoxin (U. S. P. Reference Standard), digitoxigenin (Sterling-Winthrop Research Institute), gitoxigenin (Sterling-Winthrop Research Institute), and digoxigenin (Burroughs Wellcome and Co.). Digitoxose (Hoffmann-LaRoche and Co.) was also examined. Each substance was used as received in its original container and for this preliminary study it seemed desirable, particularly with respect to the practical significance of the data obtained, to postpone the purification procedures which will be required ultimately.

The Perkin-Elmer model 137 infracord was used for scanning the absorption spectra between  $2.5\mu$  and  $15\mu$ , and the potassium bromide disk technique was employed for the preparation of the samples. Potassium bromide, infrared quality, was obtained from the Harshaw Chemical Co. For each glycosidal substance, disks were prepared on the basis of 2 mg. of substance to 300 mg. of potassium bromide. In the case of digitoxose, this ratio was 1 mg. to 300 mg. To the weighed amount of substance, which was transferred to an agate mortar, was added a small portion of the 300 mg. of potassium bromide. After this mixture was thoroughly ground by hand, the balance of the potassium bromide was added, and the final treatment was directed toward thorough mixing. The final mixture was transferred to a die (No. H-920, Hilger and Watts Ltd.) which was connected to a Cenco Hyvac pump. After a period of three minutes of evacuation, the die was compressed with a laboratory model Carver hydraulic press for a period of one minute at its maximum pressure (20,000 pounds load). The disk thickness was approximately 1 mm. In the preparation of the disks every effort was made to standardize the technique on a qualitative and quantitative basis so that the comparative value of the results could be maintained at as high a level as possible. Several disks were prepared for each substance and their spectra were scanned. Typical absorption spectra are shown in Figs. 1 to 4.

## DISCUSSION

The structural interrelationships of the digitalis compounds examined are shown in Table I. The carbohydrate moiety of the lanatosides is terminated by the glucose molecule which is attached to an acetylated digitoxose molecule preceded by two additional digitoxose molecules. The absorption spectra of the lanatosides which contain the complete sugar moiety and therefore have the largest molecular weight are shown in Fig. 1. These three absorption spectra are strikingly similar both quantitatively and qualitatively. This is particularly true in the spectra of lanatosides A and B in which the only structural difference (Table I) consists of the presence of an additional hydroxyl group at C 16 in the lanatoside B molecule. The structure of the lanatoside C molecule is characterized by the presence of an hydroxyl group at a new position at C 12. All three spectra display a high degree of absorption in the region between  $9\mu$  and  $10\mu$ .

Deacetylation and removal of the glucose molecule from the lanatosides result in the formation of the glycosides: digitoxin, gitoxin, and digoxin. These spectra, as shown in Fig. 2, reveal similar

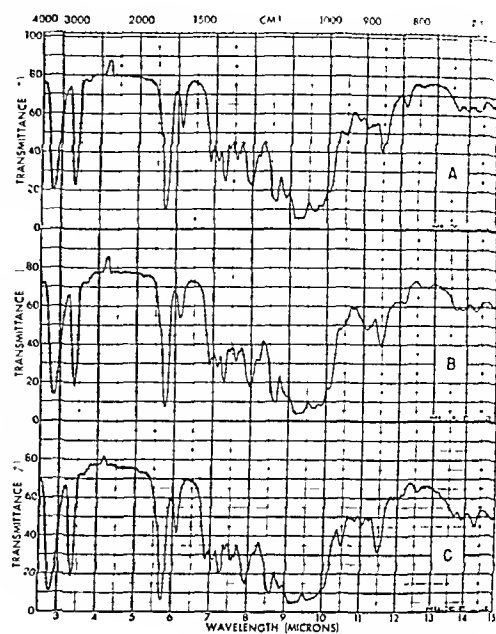


Fig. 1.—A, lanatoside A; B, lanatoside B; C, lanatoside C.

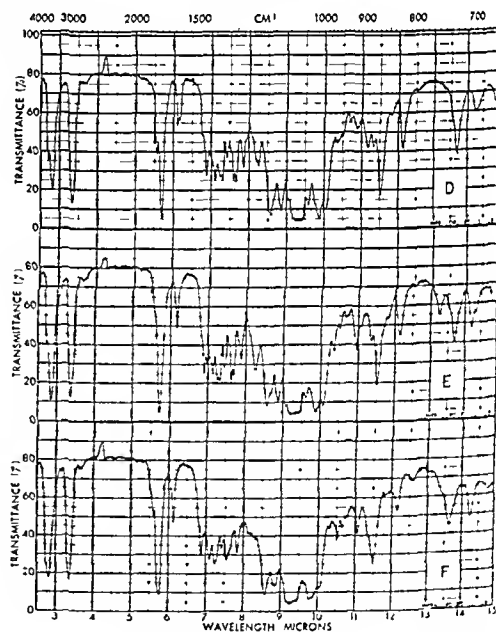


Fig. 2.—D, digitoxin; E, gitoxin; F, digoxin.

absorption patterns in the longer wavelengths at  $12.1\mu$  and  $14.2\mu$ , and also at  $11.5\mu$  and  $13.7\mu$ , at which points the absorption of digitoxose (Fig. 4) at  $11.4\mu$  and  $13.8\mu$  may be a contributing factor. The region of high absorption of the lanatosides between  $9\mu$  and  $10\mu$  is retained and perhaps extended somewhat toward the shorter wavelengths. It is worthy of mention that while gitoxin and digoxin show a very sharp absorption of medium intensity in the region of  $6.1\mu$ , in the spectrum of digitoxin, two bands appear. We have examined five other

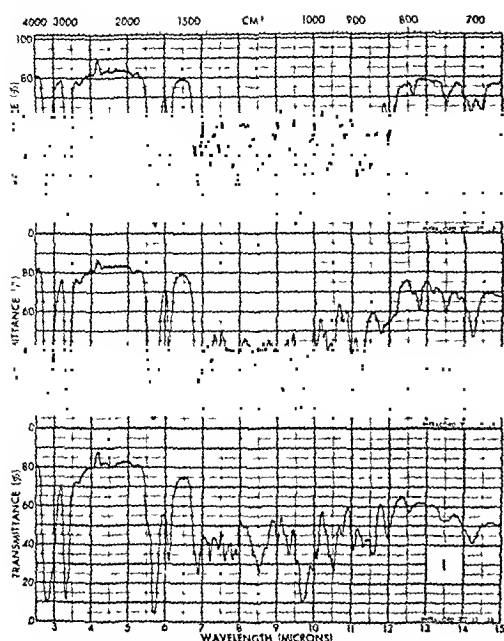


Fig. 3.—G, digitoxigenin; H, gitoxigenin; I, digoxigenin.

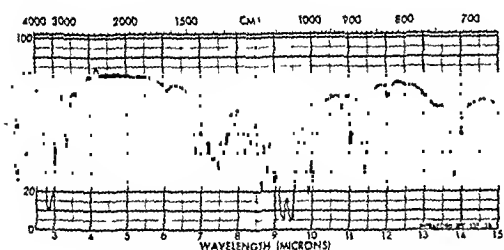


Fig. 4.—Digitoxose.

samples of this glycoside and have found, consistently, either a resolution of two bands or a single band definitely broader than that shown by gitoxin and digoxin.

The absorption spectra of the three aglycones (digitoxigenin, gitoxigenin, and digoxigenin) are shown in Fig. 3. With the complete removal of the sugar moiety there is a marked change in the resolution and transparency in the region between  $9\mu$  and  $10\mu$ , indicating again the possible influence of the presence of digitoxose in the glycosides on the spectra in this region. The sharp absorption at  $6.1\mu$  appears to reach a maximum value in these three genins.

## CONCLUSIONS

Although the question of the purity of the substances examined in this study is unanswered, there appear to be a number of conclusions which may be drawn, at least tentatively, and some of these may have some practical significance. There is a striking similarity, both qualitatively and quantitatively, in all nine spectra of the glycosidal substances in the shorter wavelength region below  $7\mu$  in spite of pronounced differences in molecular weight (Table I). Since all of the disks of these substances were prepared on the same weight basis, this is probably attributable to the diminution of the cumulative effect of the well known absorbing vibrations in this region accompanying the decrease in molecular weight.

It is clear that the experimental method and instrumentation employed are not satisfactory for the identification of the three lanatosides, particularly lanatosides A and B. There is a definite indication, however, that a closer study of the region between  $10.5\mu$  and  $11.5\mu$  might be fruitful in this connection.

On passing to the absorption spectra of the intermediate glycosides (digitoxin, gitoxin, and digoxin) minor differences appear which may be suitable for purposes of identification. It seems probable that with the diminution of the carbohydrate moiety of the lanatosides, the positions and the number of hydroxyl groups in the steroid nucleus of these substances assume a greater influence on the absorption pattern. Once again, the region between  $10.5\mu$  and  $11.5\mu$  appears worthy of further study. The absorption of these substances in the region between  $13\mu$  and  $15\mu$  contrasts markedly with that of the lanatosides and, to a lesser degree, with that of the aglycones.

In the absorption spectra of the aglycones numerous minor differences appear which may be useful for analytical purposes. With these substances, consisting of the steroid nucleus alone, the number and positions of the hydroxyl groups would be expected to exert their maximum influence on the absorption which is in accord with the experimental findings.

TABLE I.—THE STRUCTURAL INTERRELATIONSHIPS OF THE DIGITALIS COMPOUNDS EXAMINED

Substance	Mol. Wt.	Aglycone	(Digitoxose) <sub>1</sub>	Acetyl	Glucose	OH Groups
Lanatoside A	969.1	Digitoxigenin	+	+	+	C <sub>11</sub>
Lanatoside B	985.1	Gitoxigenin	+	+	+	C <sub>14</sub> C <sub>16</sub>
Lanatoside C	985.1	Digoxigenin	+	+	+	C <sub>12</sub> C <sub>14</sub>
Digitoxin	764.9	Digitoxigenin	+	...	...	C <sub>11</sub>
Gitoxin	780.9	Gitoxigenin	+	...	...	C <sub>14</sub> C <sub>16</sub>
Digoxin	780.9	Digoxigenin	+	...	...	C <sub>12</sub> C <sub>14</sub>
Digitoxigenin	374.5	....	...	...	...	C <sub>3</sub> C <sub>14</sub>
Gitoxigenin	390.5	....	...	...	...	C <sub>3</sub> C <sub>14</sub> C <sub>16</sub>
Digoxigenin	390.5	....	...	...	...	C <sub>3</sub> C <sub>12</sub> C <sub>14</sub>

The nine absorption spectra presented in this study indicate clearly that the three lanatosides can be readily differentiated from the corresponding intermediate glycosides (digitoxin, gitoxin, and digoxin) and from their aglycones by the infrared method. Furthermore, the aglycones can likewise be readily differentiated from the intermediate glycosides and from the lanatosides. Although there are a number of satisfactory chemical tests for the identification of carbohydrates, there may be instances in which the

infrared technique may be preferred, especially since the test sample is not necessarily destroyed. The results also show that the three Reference Standard glycosides (lanatoside C, digitoxin, and digoxin) can be differentiated by their infrared absorption.

## REFERENCE

(1) Dobriner, K., Katzenellenbogen, E. R., and Jones, R. N., "Infrared Absorption Spectra of Steroids," Interscience Publishers, Inc., New York, N. Y., 1953.

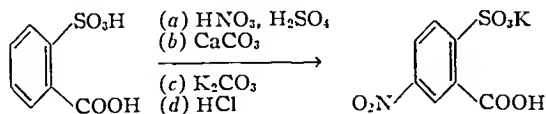
# Saccharin Derivatives II\*

## Synthesis of 4-Nitrosaccharin and Related Compounds

By GLENN H. HAMOR

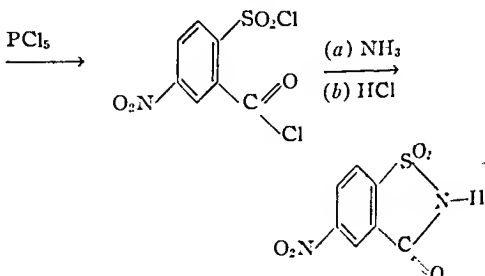
Five new saccharin derivatives were prepared: 4-nitrosaccharin, 4-aminosaccharin, 4-acetamidossaccharin, 2-ethyl-4-nitrosaccharin, and 2-*n*-propyl-4-nitrosaccharin. Attempts to prepare 5-nitrosaccharin, using procedures described in the literature, gave instead a mixture of two nitrosaccharins, which was shown to consist of the known 6-nitrosaccharin plus 4-nitrosaccharin identical to the above compound. The new saccharin derivatives were tasteless, with the exception of 4-aminosaccharin which had a slightly sweet taste.

IN A RECENT STUDY of saccharin derivatives, the preparation of various nitrosaccharins and related compounds was desired (1). 6-Nitrosaccharin was synthesized by the method of Noyes (2). Only two references have been noted in the literature in regard to saccharins containing nitro or amino groups in positions other than the 6-position. Both of these concerned the 5-position. Stubbs in 1913 (work done in 1892) reported the synthesis of 5-nitrosaccharin (3). This compound was prepared by the nitration of *o*-sulfobenzoic acid by the following series of reactions:



No melting point or percentage yield figures were given for the reported nitrosaccharin, but because the compound was more soluble than 6-nitrosaccharin Stubbs believed that he had synthesized a new nitrosaccharin. The 5-position was chosen because Taverne, in 1906, had nitrated *o*-sulfobenzoic acid and had reported obtaining 5-nitro-*o*-sulfobenzoic acid (4). The physical properties of the barium salt of Taverne's acid resembled those of the barium nitro-*o*-sulfobenzoate prepared by Stubbs.

On the basis of the above similarities Stubbs reported his nitrosaccharin as being 5-nitrosaccharin.



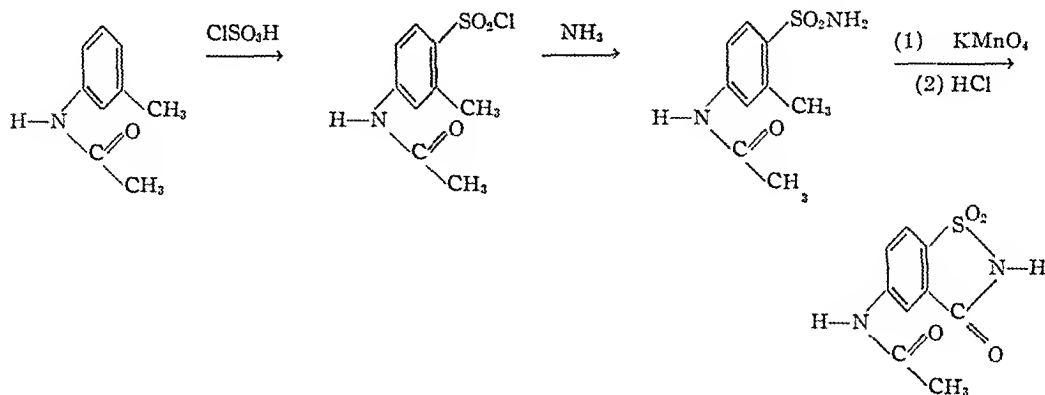
\* Received August 28, 1959, from the School of Pharmacy, University of Southern California, Los Angeles.

A portion of this work was completed at the College of Pharmacy, University of Minnesota, Minn., with the help of Dr. T. O. Soine.

rin. Suter, who has thoroughly reviewed the synthesis, reactions, and derivatives of saccharin does not include this compound (5). Bambas (6)

in his review of saccharin chemistry does list the 5-nitrosaccharin of Stubbs.

Backeberg and Marais in 1943 reported the preparation of 5-acetamidossaccharin (7) by the following plan:



Their original objective had been the synthesis of 5-aminosaccharin, because it contains the structural elements of sulfanilamide. The authors reported that the attempted deacetylation of the 5-acetamido compound resulted in a syrup from which a crystalline product could not be obtained.

Hamor (8) has reported an attempt to prepare 5-nitrosaccharin by following essentially the procedure of Stubbs. Ammonium hydrogen *o*-sulfobenzoate was converted to the barium salt with barium hydroxide and then by the action of sulfuric acid, *o*-sulfobenzoic acid was obtained. The nitration product of this acid was obtained as the crystalline barium salt. A dilute aqueous solution of the barium salt was passed through a column of cation exchange resin and gave a soft tan-colored solid, melting in the range of 90–125°. A mixture of nitration products was evidently present. It was thought that by proceeding directly to the mixed nitrosaccharins, compounds might be obtained which would differ enough in their solubilities to be readily separated. The diacid chloride of the mixed nitro-*o*-sulfobenzoic acids was prepared by heating barium nitro-*o*-sulfobenzoate with phosphorus pentachloride. The diacid chloride was not isolated as such but was extracted by ether. The mixed nitrosaccharins were obtained as the precipitated ammonium salts by passing a stream of dry ammonia gas into the dried ethereal solution of the diacid chloride.

Conversion of the ammonium nitrosaccharins to the free nitrosaccharin gave a mixture of nitrosaccharins melting in the range of 196–210°. From this mixture it was possible to isolate two compounds by the difference in solubilities of their sodium salts: (a) A light green-colored

compound possessing a slightly sweet taste and melting at 227–228° was obtained in 10% yield (for two steps), and (b) light yellow compound having a bitter taste and melting at 206–206.5° was obtained in a 3% yield. The sodium salt of

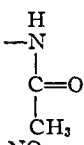
this compound was more water-soluble than the salt of the preceding compound.

The melting point of 206–206.5° for the second compound was close to that of 6-nitrosaccharin; the compound likewise resembled it in taste and color. A mixed melting point of the two compounds showed no depression. This indicates that one position nitrated in the *o*-sulfobenzoic acid had been the 4-position, which is *para* to the carboxy group and *meta* to the sulfo group.

An investigation of the other nitrosaccharin to determine, if possible, its structure was then begun. Its melting point was close to that of saccharin (228°), but a mixed melting point of the two exhibited a depression of 40°. If the nitration of *o*-sulfobenzoic acid in this investigation were analogous to that reported by Taverne (4), this unidentified nitrosaccharin should be 5-nitrosaccharin. Backeberg and Marais (7) had reported the synthesis of 5-acetamidossaccharin (m. p. 299°) by a different route. For purposes of comparison the unidentified nitrosaccharin [hereafter referred to as (?) -nitrosaccharin] was reduced to (?) -aminosaccharin and acetylated to yield (?) -acetamidossaccharin [m. p. 332–333° (decompn.)]. A mixture of the two acetamidossaccharins showed a depressed melting point in the range of 285–290° (decompn.).

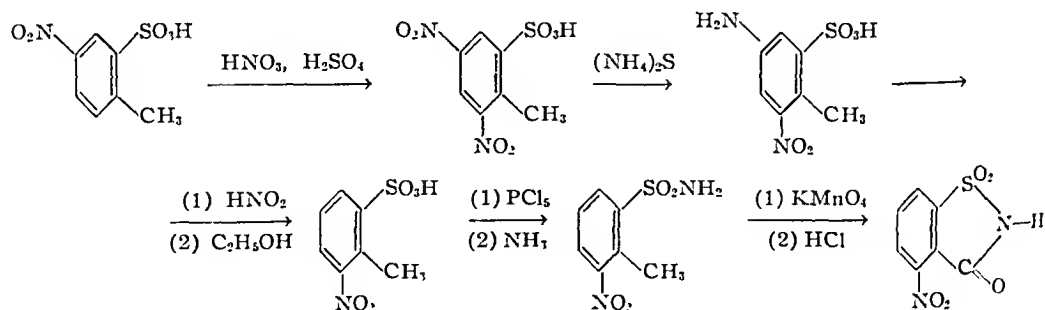
Because the unidentified acetamidossaccharin differs from that of Backeberg and Marais, the possibility that the nitro group is in the 5-position seems to be excluded. Because the 6-nitrosaccharin is well established, this leaves only the 4- and 7-positions of saccharin open for consideration. The 2-ethyl- and 2-*n*-propyl-(?) -nitrosaccharins were prepared for reference compounds by use of previously published procedures (1).

TABLE I—PROPERTIES OF 4-NITROSACCHARIN AND THE UNIDENTIFIED NITROSACCHARIN SYNTHESIZED BY THE METHOD OF STUBBS, AND DERIVATIVES

R	Y	M P °C	Analyses <sup>b</sup>				M P °C	Carbon, % Found	Hydrogen, % Found
			Calcd	% Found	Calcd	% Found			
H	NO <sub>2</sub>	236–238	36.84	36.55	1.77	1.92	241–242, mixed melt with 4-nitrosaccharin, no depression	36.88	1.80
H	NH <sub>2</sub>	246–247	42.42	42.55	3.12	3.18	248–249, mixed melt, no depression	42.55	3.24
H		323–325 <sup>d</sup> (decompn)	45.00	44.96	3.36	3.38	332–333 <sup>d</sup> decompn mixed melt, no depression	45.06	3.65
C <sub>2</sub> H <sub>5</sub>	NO <sub>2</sub>	184.5–185	42.19	42.46	3.15	3.42	184.5–185, mixed melt, no depression	42.67	3.56
C <sub>3</sub> H <sub>7</sub>	NO <sub>2</sub>	138–138.5	44.44	44.55	3.73	4.02	147–149, mixed melt, no depression	44.77	4.14

<sup>a</sup> Melting points were taken with a Fisher Johns melting point apparatus and are uncorrected<sup>b</sup> Analyses were performed by Elek Micro Analytical Laboratories, Los Angeles, Calif<sup>c</sup> Analyses were performed by Organic Microanalysis Laboratory, School of Chemistry University of Minnesota, Minneapolis<sup>d</sup> Melting point done by capillary tube method

4-Nitrosaccharin was then prepared by oxidation of 2-nitrotoluene-6-sulfonamide, which had been synthesized by the method of Hirve and Jambhekar (9), according to the following series of reactions:



Reduction of 4-nitrosaccharin, followed by acetylation gave 4-acetamidosaaccharin [m p 323–325° (decompn)]. A mixture of this compound with the 5-acetamidosaaccharin of Backeberg and Marais<sup>1</sup> melted at 282° (decompn).

<sup>1</sup> The author is indebted to J. L. C. Marais, University of Witwatersrand, Johannesburg, South Africa, for supplying the sample of 5-acetamidosaaccharin.

The 2-ethyl- and 2-*n*-propyl-4-nitrosaccharins were also prepared. The properties of 4-nitrosaccharin, (?)-nitrosaccharin, and their derivatives are shown for comparison in Table I. Mixed melting points of 4-nitrosaccharin and 4-nitrosac-

charin derivatives with the unidentified nitrosaccharin and its derivatives showed no depression. In addition, infrared absorption spectra of the two nitrosaccharins were identical.

The above data indicate that the unidentified nitrosaccharin obtained by the method of Stubbs is identical with 4-nitrosaccharin as prepared by

the oxidation of 2-nitrotoluene-6-sulfonamide. Therefore, attempts to prepare 5-nitrosaccharin following the method of Stubbs have given instead a mixture of 4-nitrosaccharin and 6-nitrosaccharin

It may be of interest to note that these new nitrosaccharin derivatives were tasteless, with the exception of 4-aminonitrosaccharin which had a slightly sweet taste.

## EXPERIMENTAL

**4-Nitrosaccharin.**<sup>2</sup>—This compound was prepared by alkaline potassium permanganate oxidation of 2-nitrotoluenc-6-sulfonamide by the method used by Noyes (2) to synthesize 6-nitrosaccharin. The method of Hirwe and Jambhekar was used to synthesize 2-nitrotoluenc-6-sulfonamide, m p 162°, (reported m p 165°) (9). Yields of approximately 30% of 4-nitrosaccharin, recrystallized from water and melting at 236–238°, were obtained. Approximately 25% of unreacted starting sulfonamide was recovered and used in succeeding oxidations.

4-Aminosaccharin.—4-Nitrosaccharin (20 Gm) in 50 ml of absolute ethanol was reduced with

<sup>2</sup> Chemical Abstract nomenclature, 4-nitro-1,2 benziso thiazolin-3-one-1,1-dioxide

hydrogen at room temperature at about three atmospheres pressure with 0.7 Gm of 5% palladium-on-charcoal catalyst. Evaporation of solvent from the filtrate and recrystallization from ethanol of the solid formed, gave 1.4 Gm of yellow crystals melting at 246–247°. Solutions of 4-aminosaccharin exhibited a beautiful blue-purple fluorescence

**4-Acetamididosaccharin.**—4-Aminosaccharin (0.8 Gm) was dissolved in 15 ml pyridine with aid of mild heat, cooled in an ice bath, and 5 ml of acetic anhydride added slowly with cooling. A cream-colored solid formed immediately. The mixture after standing forty-five minutes at room temperature, was filtered and the precipitate washed with water. A crystalline solid [0.7 Gm., 323–325° (decomn) m p] was obtained.

## REFERENCES

- (1) Hamor, G H, and Soine, T O, *THIS JOURNAL*, **43**, 120(1954)
- (2) Noyes, W A, *Am Chem J*, **8**, 167(1886)
- (3) Stubbs, M B, *ibid*, **50**, 193(1913)
- (4) Fawcett, H J, *Revue chim*, **25**, 50(1906)
- (5) Suter, C H, "The Organic Chemistry of Sulfur," John Wiley & Sons, Inc, New York, N Y, 1944, p 623
- (6) Bambar, L L, "The Chemistry of Heterocyclic Compounds," vol 4, Interscience Publishers, Inc, New York, N Y, 1952, p 350
- (7) Backeberg, O G, and Marais, J L C, *J Chem Soc*, 1943, 78
- (8) Hamor, G H, Ph D Thesis, University of Minnesota, 1952, p 36ff
- (9) Hirwe, N H, and Jambhekar, M R, *J. Indian Chem Soc*, **11**, 239(1934)

### Saccharin Derivatives III\*

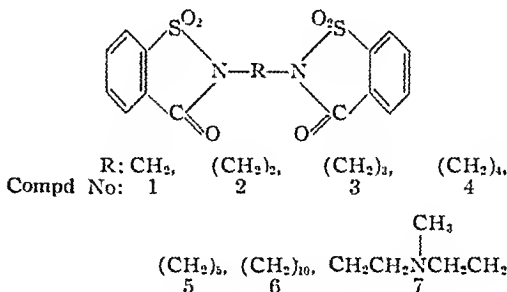
## A Note on the Synthesis of Bissaccharins

By GLENN H. HAMOR and JACK M. BALIKIAN

THE N-ALKYL and N-dialkylaminoalkyl derivatives of saccharin have a low degree of local anesthetic activity (1). The purpose of this work was the preparation of some bis-saccharins for testing of various types of biological activity, including local anesthetic properties. Such testing should give information concerning the pharmacological effects of introduction of a second saccharin moiety.

The structural formulas of the seven bissaccharins prepared are illustrated.

The saccharin derivatives were prepared according to the procedure used by Merritt, Levey, and Cutter to synthesize 2-alkylsaccharins (2). This involved refluxing sodium saccharin with an alkyl dihalide or methylbis( $\beta$ -chloroethyl)amine hydrochloride in either diethylene glycol mono-



1

ethyl ether-water or diethylene glycol monobutyl ether-water mixtures. After completion of this work the previous work of Reid, Rice, and Grogan describing the synthesis of all compounds except No. 7 (I), using dimethylformamide as the reaction medium, was seen (3). Therefore, this paper

\* Received September 15, 1959, from the School of Pharmacy, University of Southern California, Los Angeles

will describe only the results of the pharmacological evaluation and the synthesis of the one new bisaccharin

Several of the compounds have undergone preliminary pharmacological evaluation. The extreme insolubility in water of these compounds has prevented testing for local anesthetic properties. Compound No. 1 produced only an insignificant degree of increased motor activity in mice after oral doses of 1,000 to 2,000 mg/Kg. Compound No. 7 was essentially devoid of biological activity in mice following oral doses as high as 2,000 mg/Kg.<sup>1</sup> In addition, Compounds 3 and 7 have also been screened for anticancer properties. Compound 3 gave no significant reduction in animal tumor weight in Sarcoma 180 and in Carcinoma 755. Compound 7 gave no significant reduction in animal tumor weight in Sarcoma 180.<sup>2</sup>

<sup>1</sup> The authors are indebted to Smith Kline and French Laboratories, Philadelphia, Pa., for pharmacological testing.

<sup>2</sup> Anticancer screening was performed through the services of the Cancer Chemotherapy National Service Center, National Institutes of Health, Public Health Service, Bethesda, Md.

## EXPERIMENTAL

2,2' - (Methyliminodiethylene)bis(1,2 - benzothiazolin-3-one)-1,1,1',1'-tetroxide.—To 5.0 Gm of sodium saccharin in a mixture of 5 ml of distilled water and 15 ml of diethylene glycol monoethyl ether was added 0.5 Gm of methylbis( $\beta$ -chloroethyl)amine hydrochloride. After refluxing for three hours, the hot mixture was poured slowly into about 100 ml of ice water. A precipitate was formed by making the solution alkaline to excess with sodium carbonate. Recrystallization from 95% ethanol gave 0.2 Gm (17%) of white needle crystals melting at 156–158°.<sup>3</sup>

Anal.—Calcd for  $C_{19}H_{19}N_3O_6S_2$ : C, 50.88, H, 4.27. Found: C, 51.21, H, 4.44.

## REFERENCES

- (1) Hamor, G. H., and Soine, T. O., *THIS JOURNAL* 43, 120 (1954).
- (2) Merritt, L. L., Ivey, S., and Cutter, H. B., *J. Am. Chem. Soc.*, 61, 15 (1939).
- (3) Reid, E. E., Rice, L. M., and Grogan, C. H., *ibid.*, 77, 5628 (1955).

<sup>3</sup> Melting point was taken with Fisher Johns melting point apparatus and is uncorrected.

<sup>4</sup> Analyses were performed by Elck Micro Analytical Laboratories, Los Angeles, Calif.

# The Physics of Tablet Compression XII<sup>†</sup>

## Extrusion and Flow Studies on Tablet Ingredients

By R. SALISBURY<sup>†</sup> and T. HIGUCHI

A method for determining the force required to extrude materials through a hole in a tablet die by means of a high force hydraulic press is presented. Extrusion forces are given for various simple inorganic electrolytes and common tablet lubricants. Extrusion of organic tablet constituents was impossible with two exceptions. Either no extrusion could be obtained at the maximum force of the press, or implosion occurred. Mixtures of sodium chloride and potassium chloride, and sodium chloride and magnesium stearate were extruded and semiempirical equations expressing behavior were evolved. Resistance to extrusion seems to correlate with the hardness of the materials under test.

**D**URING THE FORMATION of compressed tablets under force within a die cavity, some degree of lateral flow appears to take place. Although the exact significance of the role played by such rheological behaviors, as far as the production of tablets is concerned, is presently unknown, it would appear that more information concerning flow under high pressure of granulations and other dry forms is desirable. From the standpoint of

tablet technology, it is evident, for example, that the ease with which tablets conform to the shape of the die cavity is directly dependent on the relative flowability of the material contained when under pressure.

In the present investigation, an attempt has been made to evaluate the relative flowability of compressed dry substances. As an approach in this direction the relative pressure necessary to induce extrusion of dry materials contained in a die casting through a small hole drilled normal to the direction of compression has been determined. Certain materials extruded rather smoothly when certain critical pressures were reached; others tend to expel rather violently.

\* Received August 21, 1959, from the School of Pharmacy, University of Wisconsin, Madison.

<sup>†</sup> Present address: College of Pharmacy, The Ohio State University, Columbus.

This investigation was supported in part by the American Chicle Co.

Presented to the Scientific Section, A. P. A. Cincinnati meeting, August 1959.

## EXPERIMENTAL

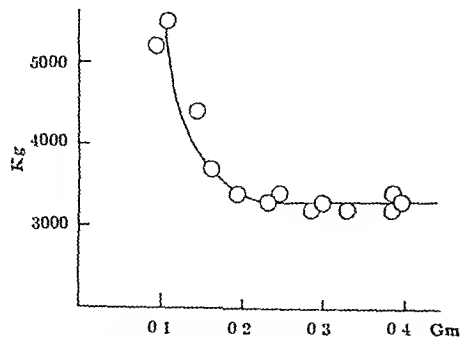
**Apparatus.**—The extrusion studies were carried out utilizing the high force hydraulic press previously described (1). This press acted on an upper punch so that it moved into a fixed die which contained a fixed lower punch. A flat face  $\frac{5}{16}$  inch punch and die set was used and, unless otherwise specified, a  $\frac{1}{16}$  inch hole was drilled in the side of the annealed die immediately above the upper edge of the fixed lower punch. The die was positioned in the assembly of the press in such a manner that the extrusion opening could be observed as pressure was applied to the upper punch.

**Procedure.**—A known weight of material was introduced into the die, and pressure applied to the upper punch so that it entered the die and compressed the experimental material. Pressure was increased slowly and regularly until the material was extruded. At the instant that the material appeared in the extrusion opening the pressure on the upper punch was released. A Baldwin type C load cell (20,000 pounds) was used to measure the force applied to the upper punch, the signal being recorded on a Sanborn "Twin-Viso" recorder. The pressure peak on the oscillograph record was taken as the extrusion force for that weight of the substance. After each extrusion run the die was removed from the assembly and the contents were removed by driving out the compacted mass using another  $\frac{5}{16}$  inch punch and a lead hammer. Punches and die were then washed in a detergent solution (a pipe cleaner serving to wash out the extrusion opening), thoroughly rinsed with distilled water, and finally rinsed with acetone, and dried in an air blast.

Preliminary experiments showed that the rate of compression had no significant effect upon the extrusion force of the particular system studied. The temperature of the room was maintained at 23°. All inorganic salts used in this experiment were dried at 110° for twelve hours and stored in a desiccator before use.

## RESULTS AND DISCUSSION

Figure 1 shows a curve of the minimum force applied to the  $\frac{5}{16}$  inch punch necessary to cause extrusion plotted against the weight of ammonium chloride introduced into the die. The abrupt increase in extrusion force for the smaller weights of material introduced is thought to be due to the mechanics of the extrusion procedure. As the

Fig 1—Extrusion force in Kg vs Gm  $\text{NH}_4\text{Cl}$ 

upper punch approaches the extrusion opening, more of its force will be transmitted to the lower punch and less to the extrusion opening. Consequently, a higher total force will be required to transmit to the extrusion opening a force sufficient for extrusion in the case of small amounts of sample. Since the outer part of the curve, representing conditions where the die was nearly full of material is at a constant force, this value was used as the extrusion force for the material undergoing compression. Reproducibility in this range of the curve was found to be  $\pm 4\%$ .

**Extrusion of Simple Inorganic Electrolytes.**—

The forces required for the extrusion of some simple inorganic electrolytes were determined. The relationships between the molecular diameter (calculated from the ionic radii measurements of Pauling) and the extrusion force show an inverse linear relationship (Fig 2). The two major deviations are ammonium bromide and ammonium chloride. All the salts tested were of the cubic type, and with the exception of ammonium bromide and chloride they were all of the sodium chloride lattice type. Ammonium bromide and chloride have a cesium chloride lattice structure at room temperature (2). This is a more stable configuration and would require a proportionately higher force for extrusion. Plots of anion radius vs extrusion force (Fig 3) and cation radius vs extrusion force (Fig 4) show the same inverse linear relationship.

The smaller the ionic radii, the stronger would be the interionic forces of attraction within the crystal and, consequently, the stronger the force necessary to cause dislocations of the planes of symmetry in the crystal which would result in solid flow.

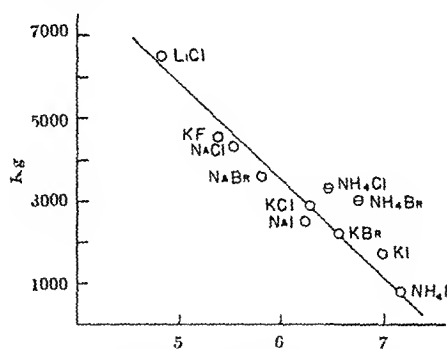


Fig 2—Extrusion forces of inorganic electrolytes vs molecular diameter

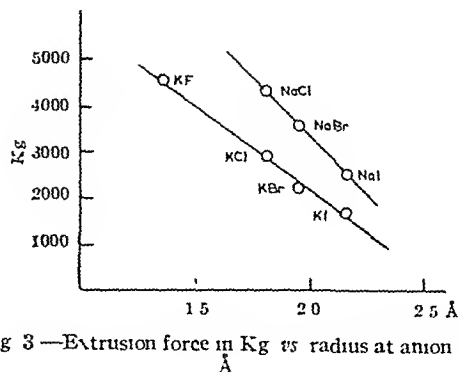


Fig 3—Extrusion force in Kg vs radius at anion in Å



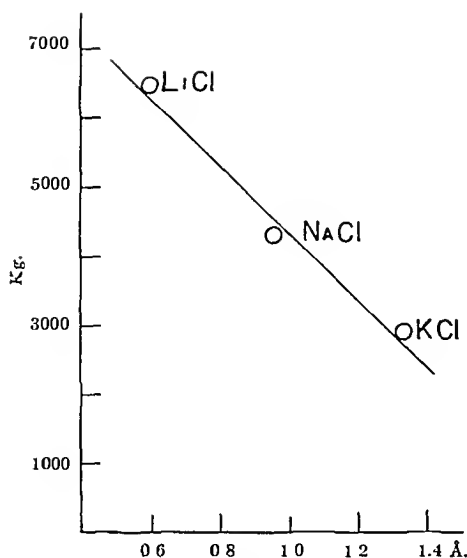


Fig. 4—Extrusion force in Kg. vs. radius at cation in Å.

**Extrusion Force vs. Radius of Extrusion Opening.**—Sodium chloride was extruded through openings of varied radius. The results are shown in Fig. 5 where the extrusion force is plotted as a function of the radius of the extrusion opening in inches. The effect, as would be expected, is an inverse linear relationship, however, the effect is not a profound one.

**Extrusion Force vs. Particle Size.**—Sodium chloride in particle size ranges varying from 10–20 mesh to 140–170 mesh were extruded and the variation in extrusion forces was well within the experimental error. Thus, particle size has no significant effect on the extrusion force. Since solid flow does not occur until considerable breakdown of the original particles has occurred, it is not surprising to find no relationship between particle size and extrusion force. The major variation was due to packing of the various particle sizes in the die and the subsequent variation in weight of sample due to efficiency of packing.

**Extrusion of Organic Materials.**—Attempts were made to extrude a variety of organic tablet components other than lubricants (see Table I). With the exception of urea and acetophenetidin, either the materials could not be extruded at forces exceeding 7,000 Kg., or, at the points listed implosion occurred.

At first the implosion was thought to be due to binding of the material within the die, but simultaneous recording of the forces on both the upper and lower punches showed simultaneous decreases in force which could not be the case if binding was the cause of the implosion. During the compression of barbitol the upper punch force was reduced by some 2,000 Kg. in 0.04 seconds. No explanation for this implosion phenomenon can be clearly demonstrated. Factors which are capable of occurring only once during a compression cycle, such as the elimination of void space, can be removed from consideration since the implosion could be repeated by recompressing the same sample. A possible explanation is the formation of a polymorph at the

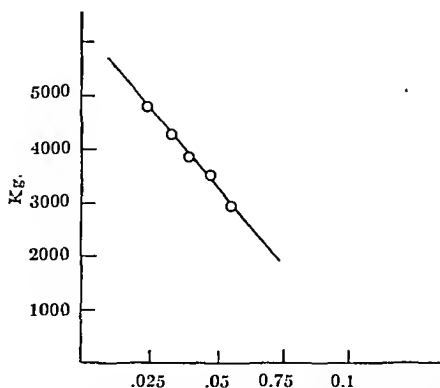


Fig. 5.—Extrusion force of NaCl vs. radius of extrusion opening in inches.

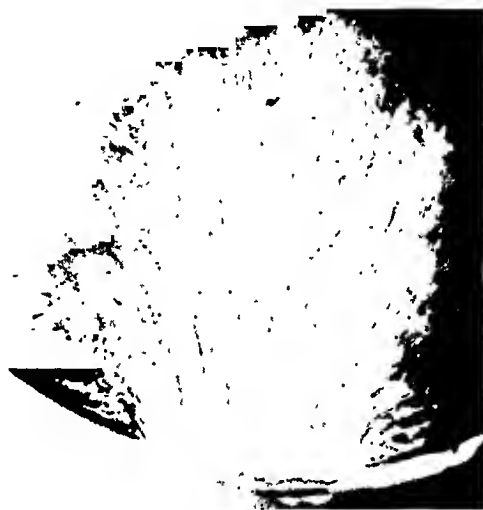


Fig. 6.—Lines of force on the lower surface of a sodium chloride sample after extrusion has occurred. The interior extrusion opening is at the bottom of the photograph and the resistance to flow is shown by the turbulence.

point of greatest resistance which is the internal opening of the extrusion hole. Figure 6 shows the lines of force formed on the lower surface of a sodium chloride sample after extrusion. The extrusion opening is shown at the bottom of the photograph and the tremendous churning effect can be seen. It is thought that at this point of highest resistance a polymorphic crystal form with a smaller molar volume might be formed resulting in a sudden drop in compressional force and that the polymorph is expelled through the extrusion opening by the resulting implosion, thus permitting the entire process to be repeated by recompression of the same sample. This theory is not borne out by the addition of lubricant to aspirin granulations (see Table I). It must be assumed that a polymorph would form at a critical pressure, and the lubricant re-

TABLE I.—BEHAVIOR OF ORGANIC COMPOUNDS TESTED UNDER HIGH FORCE COMPRESSION

Compound	Description	Behavior	Force Applied, Kg.
Dextrose	Unsieved powder	No extrusion at	7,600
Lactose	Unsieved powder	No extrusion at	7,600
Sucrose	20-Mesh crystals	No extrusion at	7,200
Barbital sodium	Unsieved powder	No extrusion at	7,200
Acetanilid	Unsieved powder	Implosion at	1,600
Ascorbic acid	20-Mesh crystals	Implosion at	6,800
Barbital	20-Mesh crystals	Implosion at	3,200
Caffeine citrate	Unsieved powder	Implosion at	6,400
Saccharin	Unsieved powder	Implosion at	3,300
Saccharin sodium	Unsieved powder	Implosion at	6,400
Sulfathiazole	Unsieved powder	Implosion at	4,100
Theophylline	Unsieved powder	Implosion at	3,000
Acetylsalicylic acid	Unsieved powder	Implosion at	2,650
Acetylsalicylic acid	20-Mesh crystals	Implosion at	2,650
Acetylsalicylic acid	Commercial granulation	Implosion at	2,650
Acetylsalicylic acid plus 1% magnesium stearate	Commercial granulation	Implosion at	2,200
Acetylsalicylic acid plus 2% magnesium stearate	Commercial granulation	Implosion at	1,700
Acetophenetidin <sup>a</sup>	Unsieved powder	Extrusion at	1,200
Urea <sup>a</sup>	Unsieved powder	Extrusion at	3,000

<sup>a</sup> One sample only.

duced the force necessary for the implosion but did not eliminate the implosion.

**Extrusion of Lubricant.**—A number of materials in common use as tablet lubricants were extruded and, as might be expected, the extrusion forces were quite low in comparison with other materials extruded. The results are listed in Table II and

TABLE II.—EXTRUSION FORCES FOR SOME COMMON LUBRICANTS<sup>a</sup>

	Kg.
Stearic acid	335
Aluminum stearate	545
Magnesium stearate	305
Calcium stearate	250
Zinc stearate	480
Talc	2,200

<sup>a</sup> As unsieved powder.

are noteworthy for the unexpectedly high force required for the extrusion of talc. If it is assumed that the lower the extrusion pressure, the more efficient the lubricant, the results are in excellent qualitative agreement with those obtained by measurement of the ejection force (3, 4). Other methods of evaluating tablet lubricants have dealt mainly with the effect of the lubricant on the interparticle friction or the "slip." Thus Nelson (5) found that magnesium stearate had no effect on the angle of repose of a tablet granulation while talc was effective in reducing it. Muenzel and Kaegi (6) also found talc to be better than the stearates in speeding the rate of flow of granules. The role of lubricants in tablet compression is apparently more complex than a simple reduction of the coefficient of friction between particles. Extrusion might be used as a measure of both "slip" and "antiadherence" effects of a lubricant.

**Extrusion of Mixtures.**—The extrusion forces for sodium chloride and potassium chloride having been demonstrated, an attempt was made to determine the variation of extrusion force with mixtures of

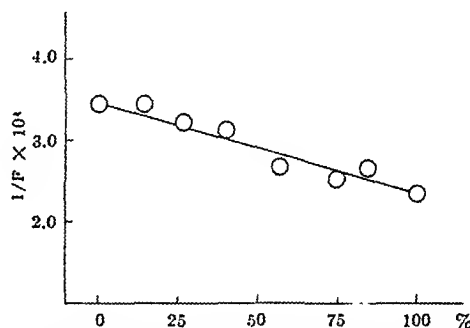


Fig. 7.—Extrusion forces of mixtures of NaCl and KCl vs. % NaCl.

the two salts. Accordingly, extrusion forces of mixtures of the two salts were determined. In Fig. 7, the values of the reciprocal of the extrusion forces are plotted against the percentage of sodium chloride present in the samples. The result is apparently a simple dilution phenomenon and the curve in Fig. 7 is calculated from the equation:

$$\frac{1}{F_t} = \frac{\% \text{ NaCl}}{100 F^{\circ} \text{ NaCl}} + \frac{\% \text{ KCl}}{100 F^{\circ} \text{ KCl}} \quad (\text{Eq. 1})$$

where  $F_t$  is the extrusion force for the mixture and  $F^{\circ}$  represents the extrusion forces of the pure components.

When the effect of adding a lubricant to sodium chloride was tested the results, when plotted as the reciprocal of the extrusion force vs. the percentage of magnesium stearate (Fig. 8), show a deviation from the semiempirical equation, Eq. 1. This deviation is especially marked in the low concentrations of the lubricant. The lower curves in Figs. 8 and 9 are calculated from the simple dilution formula, Eq. 1. The upper curve in each figure is derived from the equation:

$$\frac{1}{F_t} = 2 \frac{\% A}{100 \cdot F^{\circ} A} + \frac{\% B}{100 \cdot F^{\circ} B} \quad (\text{Eq. 2})$$

where  $F_t$  is again the total extrusion force,  $A$  is magnesium stearate,  $B$  is sodium chloride, and  $F^0$  is the extrusion force of the pure component. From Fig 9 it can be seen that the reduction in extrusion force due to the addition of magnesium stearate is greater than is to be expected from simple dilution alone. Explicitly it is greater by a factor of two times the quotient ( $\%$  lubricant/ $F^0$  lubricant). Thus in addition to simple dilution there is present a "lubricating" effect which is equal in intensity to the dilution factor up to about 2% magnesium stearate. Beyond this point the "lubricating factor" is reduced and the experimental points gradually return to the dilution curve, Eq 1. This is in reasonable agreement with the maximum effective percentage of magnesium stearate usually used in tablet manufacture.

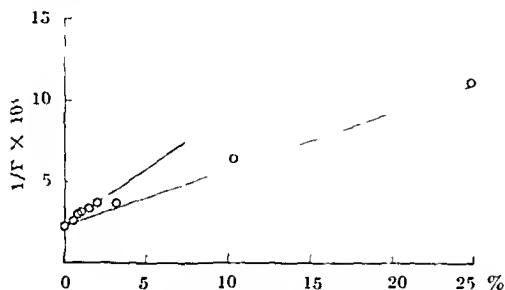


Fig 8—Extrusion forces of mixtures of NaCl and magnesium stearate vs  $\%$  magnesium stearate

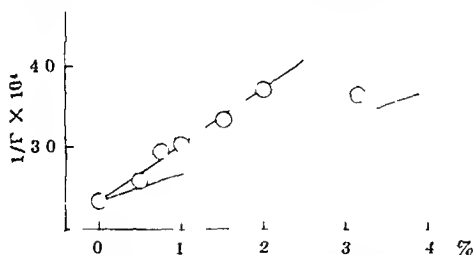


Fig 9—Extrusion forces of mixtures of NaCl and magnesium stearate vs  $\%$  magnesium stearate

It is interesting to note that Muenzel (6) found that lubricants retarded the flow of sodium chloride crystals, while in these experiments there is a definite lowering of the extrusion force. It may be postulated that the role of the lubricant in tablet compression is more than an antiadherence factor to prevent sticking to the punches and die and an increased slip factor, which enhances the flow of the

particles. It would appear from these results that the lubricant plays an additional role in decreasing the pressure necessary to obtain a desired state of compression.

Upper and lower punch force readings were recorded for several of the sodium chloride-magnesium stearate mixtures and the ratios

$$R = \frac{\text{Lower Punch Force}}{\text{Upper Punch Force}}$$

are in agreement with literature values for the addition of lubricant (3, 4). The values obtained were somewhat low since in addition to the loss of force due to die-wall resistance there is also a loss of force through the extrusion opening.

## SUMMARY AND CONCLUSIONS

1 The forces necessary for the extrusion of various inorganic electrolytes through a  $1/16$  inch hole in the side of a tablet die were measured.

2 For the most part, organic tablet constituents either did not extrude through a  $1/16$  inch opening at pressures exceeding 100 tons per square inch, or implosions occurred due to some unknown cause. Of the organic materials tested only urea and acetophenetidin could be extruded.

3 A series of tablet lubricants were extruded and their extrusion forces are in approximate order of their efficiencies as tablet lubricants.

4 The extrusion forces for mixtures of sodium chloride and potassium chloride were found to vary according to a semiempirical formula (Eq 1).

5 The extrusion forces for mixtures of sodium chloride and magnesium stearate were found to deviate from Eq 1. The deviation was twice the factor representing magnesium stearate in the right side of Eq 1 up to a lubricant concentration of 2%.

## REFERENCES

- (1) Nelson E. *This Journal* 44, 194 (1955).
- (2) Wells A. F. *Structural Inorganic Chemistry*. Oxford Press London England 1950 p 271.
- (3) Nelson E. *This Journal* 43, 566 (1951).
- (4) Strickland W. A. Jr. *et al* *ibid* 45, 51 (1956).
- (5) Nelson E. *ibid* 44, 435 (1955).
- (6) Muenzel K. and Kregi W. *Pharm Acta Helv* 29, 53 (1954).

# Esters of Bicyclic Aminoalcohols II\*

## The Synthesis of the Hydroxyquinolizidines and Some of Their Esters as Potential Therapeutic Agents

By RAYMOND E. COUNSELL† and TAITO O. SOINE

Synthesis of esters of 1- and 3-hydroxyquinolizidine as well as of the parent aminoalcohols is described, and preliminary screening suggests that antispasmodic activity is enhanced by the *beta*-arrangement. Suitable esters of all three aminoalcohols have also been synthesized to determine the degree of local anesthetic activity associated with them. A significant degree of inhibition of spontaneous motor activity in rats was noted with the trimethoxybenzoates of 1- and 3-hydroxyquinolizidine.

A NUMBER OF SYNTHETIC DRUGS have been prepared by modification of naturally-occurring compounds. In particular, cocaine and atropine have served as prototypes for the development of numerous synthetic analogs with the hope of diminishing undesirable side effects associated with the natural products. Because these natural products are esters of complex cyclic aminoalcohols, most of the investigations to date have involved variation of the acyl portion and simplification of the aminoalcohol portion of the molecules. As a result of such investigations, a number of therapeutically useful compounds derived from rather complex acids and simple acyclic aminoalcohols have been synthesized. Nevertheless, there still appears to be room for substantial improvement in this field (1).

Sternbach and Kaiser (2) have indicated that the more complex aminoalcohols of the bicyclic type may have some merit. The aminoalcohols from which they prepared esters possessed a bridgehead nitrogen and included 3-quinuclidinol, 1-azabicyclo[3.2.1]-6-octanol, 1-azabicyclo[3.3.1]-4-nonanol, and octahydro-1-pyrrocolinol. The fluorene-9-carboxylate ester of 3-quinuclidinol was twice as active as atropine in abolishing acetylcholine-induced spasm and the diphenylacetate was equivalent to atropine. Further pharmacological data reported by Randall and co-workers (3) indicated that maximal activity was obtained in compounds wherein the nitrogen and alcoholic oxygen functions of the aminoalcohol were separated by two carbon atoms. Because of the paucity of information on esters of bicyclic aminoalcohols, Rhodes and Soine (4) were prompted to investigate the antispasmodic potentiality of esters of

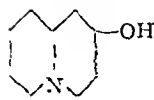
2-hydroxyquinolizidine (I). This particular aminoalcohol was chosen because of its structural relationship to tropinol (II) in so far as the relative positions of the nitrogen and alcoholic functions are concerned. The only compound with significant atropine-like activity in this series, however, was the methobromide of the ester derived from xanthene-9-carboxylic acid. Nevertheless, in light of the findings of Randall and co-workers on esters of quinuclidinol (III) the preparation of esters of 1- and 3-hydroxyquinolizidine (IV and V) was indicated in the hope of potentiating the pharmacologic activity of this series. These two aminoalcohols would be isomeric with the previously examined compound but would differ in the essential aspect that each would have the desired *beta*-relationship of the nitrogen and alcoholic functions. Consequently, some esters of these two aminoalcohols have been prepared for pharmacological testing as antispasmodics or anticholinergics and, in addition, other esters of both alcohols as well as of 2-hydroxyquinolizidine have been prepared for evaluation as local anesthetics. In this latter respect, the structural relationship of piperocaine<sup>1</sup> (VI) [ $\gamma$ -(2-methylpiperidine)-propyl benzoate hydrochloride] to the benzoate of 2-hydroxyquinolizidine (VII) is worthy of note. In a study of piperidine and substituted piperidine-alkyl benzoates, it was noted by McElvain (5) that only the substituted compounds exerted any anesthetic effect on mucous membranes. The favorable pharmacological properties of piperocaine have led to its rather extensive clinical acceptance for both topical and infiltration anesthesia. Moreover, a number of compounds have been synthesized involving minor variations of the piperocaine molecule (6). A close inspection of VII reveals that it may be looked upon as a closed ring form of VI. Since an *alpha*-methyl group

\* Received August 21, 1959, from the College of Pharmacy, University of Minnesota, Minneapolis.  
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

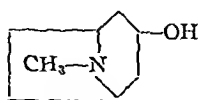
† Samuel W. Melendy Fellow, 1955-1957. Present address: G. D. Searle and Co., Chicago, Ill.

Gratitude is also expressed to the Canadian Foundation for the Advancement of Pharmacy for financial assistance.

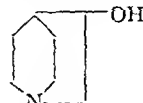
<sup>1</sup> Piperocaine is marketed under the brand name of Metycaine.



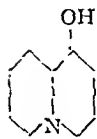
I



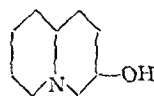
II



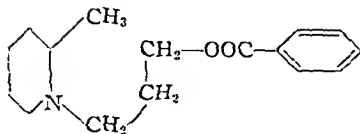
III



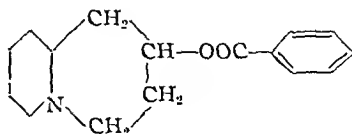
IV



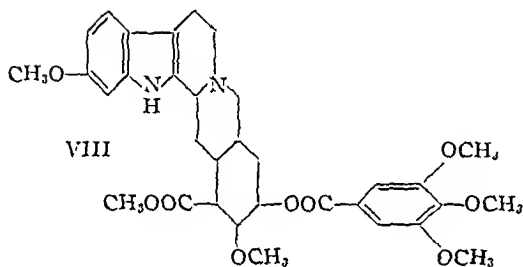
V



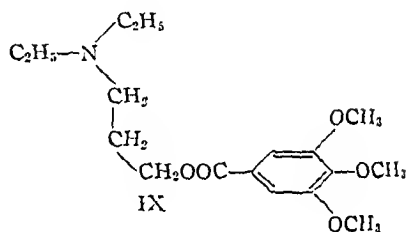
VI



VII



VIII



IX

on the piperidine ring increases activity, it seemed of interest to determine what effect spatial restriction, in the form of another ring, would have upon the activity of the compound. Consequently, the preparation of the benzoates of the three isomeric aminoalcohols was indicated.

Compounds containing the quinolizidine ring structure are of further interest since this nucleus is found in a number of naturally occurring alkaloids. Considering the extensive interest in reserpine (VIII), preparation of the trimethoxybenzoates of the three aminoalcohols seemed inviting. The same procedures of chemical dissection reminiscent of the tropane alkaloids, cocaine and atropine, have been applied to reserpine by Miller and Weinberg (7, 8) with the result that the  $\gamma$ -dichethylaminopropyl ester of trimethoxybenzoate (IX) was shown to have one-third the activity of reserpine in the tests employed.

A search of the literature revealed that all three aminoalcohols had been synthesized previously. Although the 2- and 3-hydroxyquinolizidines were reported only recently (9, 10), the corresponding ketones of all three aminoalcohols have been known for some time. These were prepared by Clemo and co-workers (11-13), via the Dieckmann cyclization reaction, during the course of an extensive investigation of the lupine alkaloids.

Although the Dieckmann cyclization also is utilized in this work there are enough useful modifications in procedure, resulting in enhanced yields, that these should be mentioned.

The synthesis of IV reported in the present paper is illustrated by Fig. 1. The Clemo synthesis of IV is improved considerably by the adoption of Reckhow and Tarbell's procedure (14) of alkylating ethyl piperidine-2-carboxylate with ethyl  $\gamma$ -bromobutyrate (easily obtained from  $\gamma$ -butyrolactone) instead of with the corresponding nitrile. These workers also reported an improved method for preparing ethyl piperidine-2-carboxylate which in turn has been improved in the present report. A marked improvement over the reported procedures for preparing the diester was noted when the alkylation reaction was carried out by reacting two equivalents of amino-ester for each equivalent of bromoester rather than carrying out the reaction with equimolar quantities in the presence of anhydrous potassium carbonate.

The synthesis of I was carried out in essentially the same manner and in the same yields as described by Rhodes and Soine (Fig. 2) with the principal difference being that the cyclization was effected more conveniently with sodium hydride rather than metallic sodium.

Figure 3 illustrates the presently reported

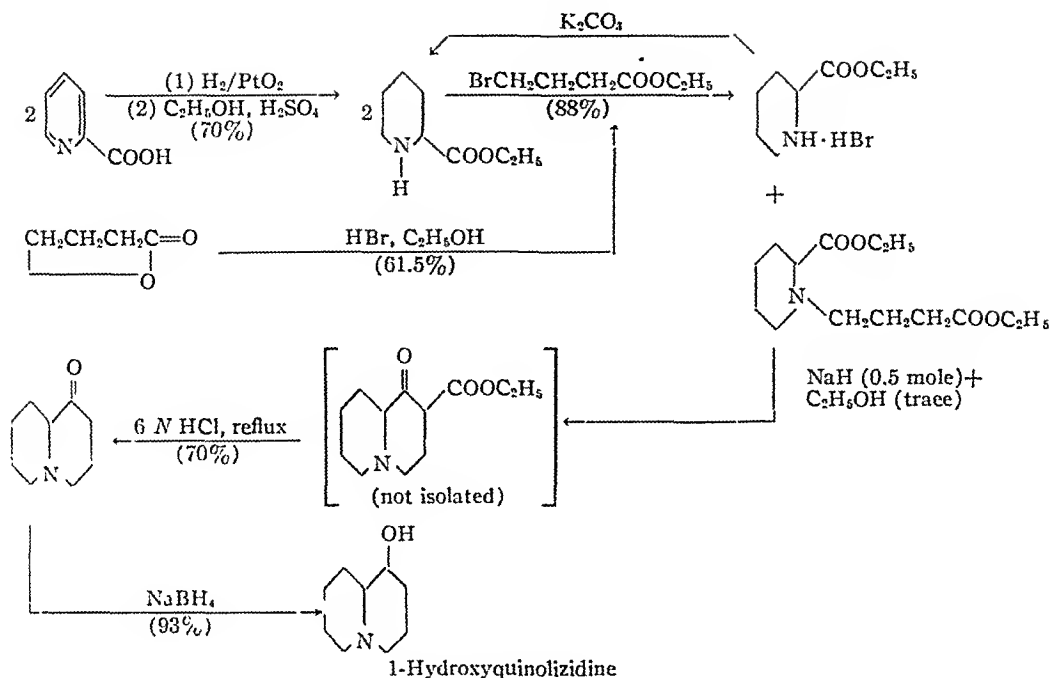


Figure 1.

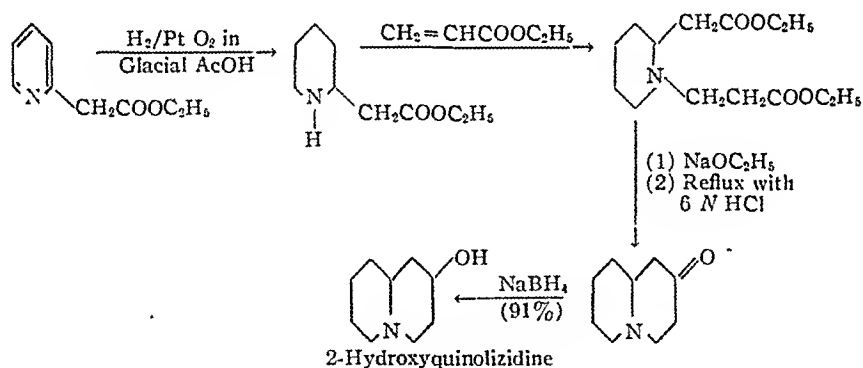


Figure 2.

synthesis of V. The method of Clemo posed a somewhat formidable problem with respect to the acquisition of sufficient diester for the cyclization reaction. These workers resorted to a somewhat lengthy procedure of condensation, hydrolysis, esterification, and hydrogenation to obtain ethyl- $\beta$ -pyridyl propionate. To achieve the same goal, the procedure of Boeckelheide (15) was used to obtain  $\beta$ -(2-pyridyl)-propionitrile by interaction of 2-vinylpyridine with acetic anhydride and potassium cyanide followed by ethan-olysis of the nitrile to the desired ethyl ester. Another desirable modification of procedure was in the use of sodium hydride as a convenient condensing agent rather than either potassium or sodium metal.

In all cases the smooth reduction of the keto-quinolizidines was most easily accomplished by the use of sodium borohydride and, in analogy to other such reductions, the product is expected to be the epimer with the hydroxyl group having the equatorial conformation. Indeed, the use of platinum oxide as the catalyst in the hydrogenation of 3-ketoquinolizidine, in contrast to the quantitative hydrogenation experienced with the 2-isomer (4), resulted in hydrogenolysis with the formation of substantial amounts of quinolizidine and lowered yields of the desired compound.

The acids employed in this work were either commercially available or were readily synthesized by known methods.

Three general methods were employed for the

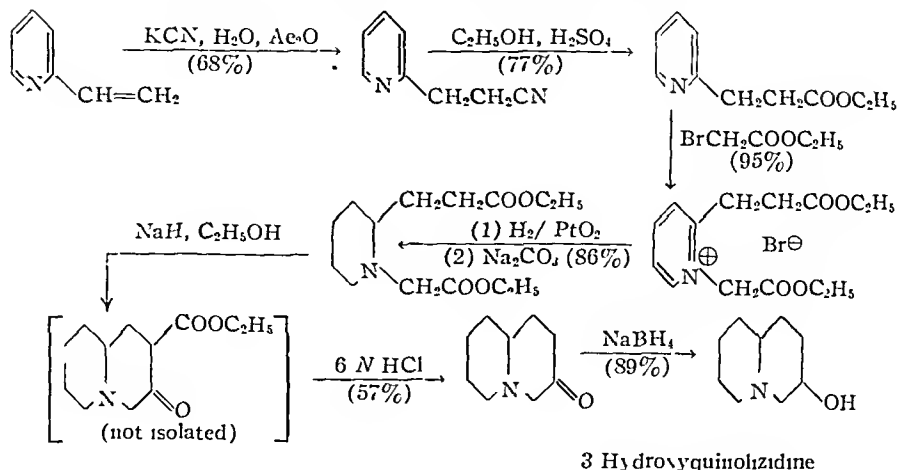


Figure 3

preparation of the esters In the preparation of the substituted acetic acid esters, the use of triethylamine (16) proved to be advantageous. This tertiary amine is sufficiently basic to be preferentially precipitated as the hydrochloride (method 1a). An alternate method (method 1b) was to use two moles of the aminoalcohol per mole of acid chloride. The acid chlorides were prepared in the usual manner with thionyl chloride and were employed in the crude form directly after removal of solvent and excess reagent. The acylations were performed in benzene with equimolar quantities of acid chloride, aminoalcohol, and triethylamine (or another mole of aminoalcohol). The base hydrochloride precipitated during the reaction and, on the basis of its weight, was useful for indicating the extent of reaction. When method 1 was applied to the preparation of the benzoate esters, the yields were generally poor and the products extremely difficult to purify. The Schotten-Baumann reaction also proved to be without value. These esters, however, were prepared by the ester-interchange reaction (method 2) in essentially quantitative yields and in a form easily purified. Although sodium alkoxides are the common catalysts for this reaction, sodium hydride was not only more convenient but also productive of higher yields. Contrary to Remer and Downes (17), who stated that under the conditions of their reactions ester interchange with methyl benzoate was restricted to primary alcohols, this reaction was found to proceed well and was definitely the method of choice for the preparation of these esters. The phenylcarbamate esters were prepared in the usual manner by treating the aminoalcohols with phenylisocyanate (method 3).

The results of antispasmodic testing<sup>2</sup> were not

as clear cut as could have been desired because the standard used (methantheline bromide) gave results indicating complete spasmolysis at a lower concentration (1:1,000,000) than partial inhibition (1:200,000). This was attributed by the pharmacologist to the vagaries of physiological test materials. However, if methantheline bromide is considered to be approximately equivalent to atropine in preventing contraction of excised ileum when stimulated by acetylcholine, the activities of some of the esters would indicate that activity of the 1- and 3-hydroxyquinolizidine esters is as great or greater than the corresponding esters of 2-hydroxyquinolizidine. The difference seems to be significant but requires more study under standardized conditions. The methobromides, as expected, showed substantially more activity than the corresponding hydrochlorides. All of the compounds tested showed more activity than papaverine hydrochloride in reducing barium-induced muscle spasms. The results of the antispasmodic testing are shown in Table IV.

Local anesthetic activity of the various esters prepared is recorded in Table V using piperocaine as the standard with an arbitrary value of 1. Activity was found to be superior to that of the standard in the xanthene-9-carboxylic acid and diphenylacetic acid esters of 3-hydroxyquinolizidine, the xanthene-9-carboxylic acid ester of 1-hydroxyquinolizidine, and in the benzoate of 2-hydroxyquinolizidine. The latter showed an activity 172 times that of the standard, indicating that the steric restriction imposed by the ring combined with optimal interprosthetic dis-

<sup>2</sup> The authors are indebted to the Rowell Laboratories, Inc., Baudette, Minn., who arranged to have the testing done by the Hazleton Laboratories, Falls Church, Va.

tance could be a factor in the activity of piperocaine. Neither of the other two benzoates was significantly active. The phenylcarbamates were of uniformly low activity.

The effects of the trimethoxybenzoates of the 1- and 3-hydroxyquinolizidines on spontaneous motor activity of rats was determined by the use of activity cages. Both of the compounds depressed spontaneous motor activity in a manner qualitatively similar to chlorpromazine and reserpine but were considerably less potent. The test, however, is not specific for tranquilizing properties inasmuch as barbiturates, narcotics, antihistamines, and a host of other drugs will produce similar results. The compounds will be studied in more detail, however, in an effort to elucidate the mechanism of action. This will be reported upon by others at a later date. Table VI gives a summary of the results of testing.

## EXPERIMENTAL

### Synthesis of Aminoalcohols

**1-Hydroxyquinolizidine.**—*Ethyl Piperidine-2-carboxylate*.—This compound was prepared from picolinic acid by hydrogenation and esterification according to the procedure of Reckhow and Tarbell (14) which employs sulfuric acid as catalyst in the latter step.<sup>3</sup> The esterification procedure was improved somewhat by employing a Soxhlet apparatus charged with a mixture of anhydrous sodium and calcium sulfates in the extraction chamber to serve as drying agents. The yield was 68% of theory, b. p. 65–70°/2 mm.,  $n_D^{20}$  1.4554. Reported, b. p. 83–85°/7 mm. (14); 92°/12 mm. (11);  $n_D^{20}$  1.4547 (18). Hydrochloride, m. p. 213.5–214°; reported, 202–203° (14).

*Ethyl  $\gamma$ -Bromobutyrate*.—This ester was prepared from butyrolactone in 61.5% yield by the method of Reckhow and Tarbell, b. p. 65–67°/4 mm.,  $n_D^{20}$  1.4555. Reported, b. p. 76–78°/7 mm.,  $n_D^{20}$  1.4538 (14) and 1.4539 (19).

*Diethyl Piperidyl-1- $\gamma$ -butyrate-2-carboxylate*.—Ethyl piperidine-2-carboxylate (142.7 Gm., 0.91 mole) and ethyl  $\gamma$ -bromobutyrate (90 Gm., 0.46 mole) were placed in a 2-L., three-necked flask and anhydrous benzene (600 ml.) added. The flask was equipped with a mercury sealed stirrer and a condenser fitted with a drying tube, and the contents were refluxed on a steam bath for forty hours, with stirring. The mixture was allowed to cool and the precipitated salt removed by filtration. After washing the precipitate with anhydrous benzene (two 200-ml. portions), the benzene was removed from the filtrate under slightly reduced pressure. During the concentration, any additional salt that formed was removed by filtration. After removal of the benzene, the product was distilled to yield 109.3 Gm. (88%), b. p. 142–145° at 1.2 mm.,  $n_D^{20}$  1.4602. Reported, b. p. 162° at 8 mm.,  $n_D^{20}$  1.4604 (14) and 1.4588 (20). Ethyl piperidine-2-carboxylate was recovered

quantitatively from the above hydrobromide salt by treatment with excess potassium carbonate solution (50% w/v), extraction with benzene, removal of solvent, and distillation under reduced pressure.

**1-Ketoquinolizidine.**—Dry nitrogen was bubbled through anhydrous xylene<sup>4</sup> (200 ml.) contained in a 1 L., three-necked flask and sodium hydride<sup>5</sup> (26 Gm. of a 45.8% dispersion, 0.5 mole) was then added. Commercial absolute ethanol (1 ml.) was added and the mixture heated to the reflux temperature. Diethyl piperidyl-1- $\gamma$ -butyrate-2-carboxylate (54 Gm., 0.2 mole), dissolved in anhydrous xylene (200 ml.), was added dropwise with stirring over a period of two hours. Stirring at the reflux temperature was continued for an additional five hours, the mixture cooled in ice, and glacial acetic acid (34 Gm., 0.57 mole) added slowly to decompose the excess hydride. The passage of nitrogen was stopped and water (100 ml.) was added to dissolve the sodium acetate formed. The aqueous layer was separated and extracted with xylene (three 25-ml. portions) which was added to the original xylene phase. The xylene layer was then extracted three times with 6 N hydrochloric acid (150-ml. portions) and the combined acid extracts heated under reflux for ten hours. After cooling, the solution was neutralized and saturated with solid potassium carbonate and the excess solids collected on the filter and washed with ether. The ether washings were then used to extract the aqueous solution (five 100-ml. portions). The combined ether extracts were dried, the ether removed by distillation, and the product collected under reduced pressure. The yield of 1-ketoquinolizidine was 21.5 Gm. (70% of theory), b. p. 65–70°/0.15–0.2 mm. [reported, 95°/4 mm. (20)],  $n_D^{20}$  1.4912 [reported  $n_D^{20}$  1.4935 (14)]. The picrate was prepared in ether and recrystallized from an ethyl acetate-cyclohexane mixture, m. p. 166–167° [reported, 167–168° (20)].

**1-Hydroxyquinolizidine.**—1-Ketoquinolizidine (21 Gm., 0.137 mole) was dissolved in water (50 ml.) and slowly added to a solution of sodium borohydride (1.5 Gm.) in water (50 ml.). The mixture was shaken and kept cool during the addition. After allowing the mixture to stand at room temperature for five hours, ammonium hydroxide solution (20 ml.) was added and the mixture allowed to stand for an additional hour. The solution was then saturated with sodium chloride and extracted with hot benzene (four 50-ml. portions). The benzene extracts were combined, dried over anhydrous sodium sulfate, and the solvent removed under slightly reduced pressure. A small portion of the white solid remaining in the flask was washed with ether and melted at 73–74° [reported, 65–68° (11)]. The remainder was distilled *in vacuo* to yield 19.7 Gm. (93% of theory) of product, b. p. 92–93°/15 mm. [reported, 127–130°/18 mm. (20)]. The picrate was prepared by precipitation from ether and recrystallization from absolute ethanol to yield yellow needles, m. p. 175.5–176.5° [reported, 174–175° (20)].

**2-Hydroxyquinolizidine.**—2-Ketoquinolizidine.—This aminoketone was prepared according to

<sup>3</sup> The use of absolute ethanol and dry hydrogen chloride gas in the esterification procedure resulted in a 12% yield.

<sup>4</sup> Redistilled and stored over calcium hydride.

<sup>5</sup> Obtained from Metal Hydrides Inc., 12-24 Congress St., Beverly 17, N. Y.



the method of Rhodes and Soine (4) in substantially the same yields as reported by these workers. The product corresponded in all physical properties and properties of derivatives with those reported earlier.

**2-Hydroxyquinolizidine.**—This compound was prepared by reduction of 2-ketoquinolizidine with sodium borohydride in the same manner as described for the preparation of 1-hydroxyquinolizidine above. Distillation of the product provided a 91% yield of a clear, colorless, viscous oil, b. p. 89–93°/0.1–0.15 mm. [reported, 132–133°/15 mm. (4)]. On cooling, the product solidified and melted at 89–90° [reported, 91–92° (4) and 92° (9)].

**3-Hydroxyquinolizidine.** —  $\beta$  - (2 - Pyridyl) - propionitrile. — This compound was prepared by the method of Boekelheide and co-workers (15) from 2-vinylpyridine, acetic anhydride, and potassium cyanide in 68% yield as an almost colorless oil, b. p. 95–96°/2 mm.,  $n_D^{25}$  1.5180 [reported, b. p. 97–99°/2 mm.,  $n_D^{25}$  1.5175 (15); b. p. 85–87°/1 mm. (21); b. p. 93–96°/1.25–1.5 mm. (22)]. The picate was prepared in ether and recrystallized from ethanol, m. p. 148–150° (decompn.) [reported, 140–142° (decompn.) (15, 22)].

**Ethyl -  $\beta$  - (2 - pyridyl) - propionate.** — Commercial absolute ethanol (500 ml.) and  $\beta$ -(2-pyridyl)-propionitrile (50 Gm., 0.38 mole) were placed in a 1-L. round-bottomed flask. Concentrated sulfuric acid (50 ml.) was then slowly added to this mixture with stirring and cooling.<sup>6</sup> The resulting solution was placed on a steam bath, fitted with a condenser and drying tube, and refluxed for eighteen hours. At the end of this time the solution was concentrated, the viscous residue dissolved in water (100 ml.), and the resulting solution basified with ice cold aqueous 15% (w/v) sodium hydroxide solution. The mixture was then immediately extracted with ether (three 150-ml. portions) and the combined ether extracts dried over anhydrous sodium sulfate. The solvent was removed and the residue fractionated under reduced pressure. The fraction distilling at 96–100°/0.9 mm. was collected to yield 52.5 Gm. (77% of theory) of a clear, colorless liquid,  $n_D^{25}$  1.4920. The reported b. p. is 95°/1 mm. (13). The picate separated as an oil from ethanol and was recrystallized from ether, m. p. 83.5–84.5° [reported, m. p. 84° (13) and 83–84° (23)].

**Diethyl Pyridinium-1-acetate-2- $\beta$ -propionate Bromide.**—Ethyl- $\beta$ -(2-pyridyl)-propionate (70.0 Gm., 0.39 mole), ethyl bromoacetate (65 Gm., 0.39 mole), and anhydrous acetone (300 ml.) were placed into a 500-ml., three-necked flask and the mixture was then refluxed for eighteen hours on a steam bath. After cooling the refluxed mixture, the separated solids were removed by filtration, washed with ether, and allowed to dry. The crude product weighed 128 Gm. (95% of theory) and was used in the subsequent reaction without further purification. Recrystallization from an acetone-ethanol mixture afforded crystals, m. p. 163–164° (decompn.) [reported, m. p. 158–159° (decompn.) (24) and 159° (decompn.) (13)].

**Diethyl Piperidyl-1-acetate-2- $\beta$ -propionate.**—Diethyl pyridinium-1-acetate-2- $\beta$ -propionate bro-

mide (20 Gm., 0.058 mole) was dissolved in water (170 ml.) and glacial acetic acid (30 ml.). The mixture was shaken with platinum oxide (0.1 Gm.) and hydrogen in a standard Parr low pressure hydrogenation apparatus. After the required amount of hydrogen had been absorbed, the mixture was filtered and the filtrate concentrated under partially reduced pressure. The resulting residue was basified with a saturated solution of sodium carbonate and immediately extracted with ether (three 125-ml. portions). The combined ether extract was dried over anhydrous sodium sulfate. After removal of the ether by distillation, the residue was fractionated under reduced pressure to yield 13.5 Gm. (86%) of a colorless liquid, b. p. 134–135°/0.9 mm.,  $n_D^{25}$  1.4634 [reported, b. p. 138–140°/1 mm. (13) and 112–113°/0.35 mm.,  $n_D^{25}$  1.4645 (24)].

**3-Ketoquinolizidine.**—The Dieckmann cyclization of diethyl piperidyl-1-acetate-2- $\beta$ -propionate was performed in a manner almost identical to that described for the preparation of 1-ketoquinolizidine (*loc. cit.*). A 57% yield of the aminoketone was realized, b. p. 63–65°/0.25 mm.,  $n_D^{25}$  1.4910 [reported, b. p. 62–63°/0.65 mm.,  $n_D^{25}$  1.4926 (24)]. The picate was prepared in ether and recrystallized from acetone at a low temperature, m. p. 180–181° (decompn.) [reported 180–182° (decompn.) (24)].

**3-Hydroxyquinolizidine.**—Reduction of 3-ketoquinolizidine with sodium borohydride in the manner described for 1-hydroxyquinolizidine (*loc. cit.*) yielded 3-hydroxyquinolizidine in 89% yield, b. p. 83–86°/0.3 mm.,  $n_D^{25}$  1.5020 [reported, b. p. 128°/14 mm. (20)]. The picate was prepared in ether and recrystallized from ethanol, m. p. 161.5–163° [reported, m. p. 161.5–162.5° (20)].

Hydrogenation of 3-ketoquinolizidine catalytically with a platinum oxide catalyst and ferrous sulfate promoter yielded a mixture of the expected 3-hydroxyquinolizidine (61% yield) and quinolizidine, b. p. 41–45°/0.6 mm.,  $n_D^{25}$  1.4830 [reported (25), b. p. 84°/21 mm.,  $n_D^{25}$  1.4796]. The picate was prepared, m. p. 196.5–199° [reported, m. p. 198–199° (25)]. The melting point of this picate was not depressed by admixture with an authentic sample of quinolizidine picate kindly supplied by Dr. V. Boekelheide.<sup>7</sup>

## Synthesis of Esters<sup>8</sup>

**Method 1 a.—Quinolizidin-3-yl Diphenylacetate Hydrochloride.**—The acid chloride prepared from diphenylacetic acid (3.0 Gm., 0.014 mole) and thionyl chloride (10 ml.) was dissolved in anhydrous benzene (20 ml.) and slowly added with stirring to a mixture of 3-hydroxyquinolizidine (2.0 Gm., 0.013 mole) and triethylamine (2 ml.) dissolved in anhydrous benzene (20 ml.). The reaction mixture was kept cool throughout the addition period. After allowing the reaction to proceed at room temperature for one-half hour the mixture was

<sup>7</sup> Department of Chemistry, University of Rochester, Rochester, N. Y.

<sup>8</sup> See Tables I, II, and III for physical constants and analytical data. Because the synthesis of the esters fell into the three general methods previously discussed they will not be treated individually and only a representative synthesis of each general category is given. The example given is typical of all syntheses in that group.

<sup>6</sup> The use of absolute ethanol and dry hydrogen chloride gas in the ethanolysis procedure resulted in a 55% yield.

TABLE I—ESTERS OF 1-HYDROXYQUINOLIZIDINE

	Method of Prepn	Recrystallized from	M p, °C	Empirical Formulas	Analyses			
					Carbon, % Calcd	Carbon, % Found	Hydrogen, % Calcd	Hydrogen, % Found
	1a	Methylene chloride, ethyl acetate, ether	161-163	C <sub>7</sub> H <sub>7</sub> NO · HCl	71 58	71 47	7 31	7 29
	1a	Isopropanol acetone, ether	234-236 (decompn)	C <sub>23</sub> H <sub>27</sub> NO <sub>2</sub> · CH <sub>3</sub> Br	64 86	64 19	6 81	6 86
		Methylene chloride, ethyl acetate	210-212	C <sub>25</sub> H <sub>19</sub> NO <sub>2</sub> · HCl	69 07	68 79	6 55	6 48
		Isopropanol, acetone, ether	225-226	C <sub>25</sub> H <sub>25</sub> NO <sub>2</sub> · CH <sub>3</sub> Br	62 89	62 67	6 16	5 97
	1b, 2	Isopropanol, isopropyl ether	205 5-206 5	C <sub>19</sub> H <sub>17</sub> NO <sub>3</sub> · HCl	59 14	59 24	7 31	7 30
	2	Ethanol, isopropyl ether, acetone	189-191	C <sub>14</sub> H <sub>21</sub> NO <sub>2</sub> · HCl	64 96	65 16	7 50	7 46
	3	Ethanol isopropyl ether	213-215	C <sub>16</sub> H <sub>17</sub> N <sub>2</sub> O <sub>2</sub> · HCl	61 83	61 88	7 46	7 33

the method of Rhodes and Soine (4) in substantially the same yields as reported by these workers. The product corresponded in all physical properties and properties of derivatives with those reported earlier.

**2-Hydroxyquinolizidine.**—This compound was prepared by reduction of 2-ketoquinolizidine with sodium borohydride in the same manner as described for the preparation of 1-hydroxyquinolizidine above. Distillation of the product provided a 91% yield of a clear, colorless, viscous oil, b. p. 89–93°/0.1–0.15 mm. [reported, 132–133°/15 mm. (4)]. On cooling, the product solidified and melted at 89–90° [reported, 91–92° (4) and 92° (9)].

**3-Hydroxyquinolizidine.**— $\beta$ -(2-Pyridyl)-propionitrile.—This compound was prepared by the method of Boeckelheide and co-workers (15) from 2-vinylpyridine, acetic anhydride, and potassium cyanide in 68% yield as an almost colorless oil, b. p. 95–96°/2 mm.,  $n_D^{25}$  1.5180 [reported, b. p. 97–99°/2 mm.,  $n_D^{25}$  1.5175 (15); b. p. 85–87°/1 mm. (21); b. p. 93–96°/1.25–1.5 mm. (22)]. The picate was prepared in ether and recrystallized from ethanol, m. p. 148–150° (decompn.) [reported, 140–142° (decompn.) (15, 22)].

**Ethyl- $\beta$ -(2-pyridyl)-propionate.**—Commercial absolute ethanol (500 ml.) and  $\beta$ -(2-pyridyl)-propionitrile (50 Gm., 0.38 mole) were placed in a 1-L. round-bottomed flask. Concentrated sulfuric acid (50 ml.) was then slowly added to this mixture with stirring and cooling.<sup>6</sup> The resulting solution was placed on a steam bath, fitted with a condenser and drying tube, and refluxed for eighteen hours. At the end of this time the solution was concentrated, the viscous residue dissolved in water (100 ml.), and the resulting solution basified with ice cold aqueous 15% (w/v) sodium hydroxide solution. The mixture was then immediately extracted with ether (three 150-ml. portions) and the combined ether extracts dried over anhydrous sodium sulfate. The solvent was removed and the residue fractionated under reduced pressure. The fraction distilling at 96–100°/0.9 mm. was collected to yield 52.5 Gm. (77% of theory) of a clear, colorless liquid,  $n_D^{25}$  1.4920. The reported b. p. is 95°/1 mm. (13). The picate separated as an oil from ethanol and was recrystallized from ether, m. p. 83.5–84.5° [reported, m. p. 84° (13) and 83–84° (23)].

**Diethyl Pyridinium-1-acetate-2- $\beta$ -propionate Bromide.**—Ethyl- $\beta$ -(2-pyridyl)-propionate (70.0 Gm., 0.39 mole), ethyl bromoacetate (65 Gm., 0.39 mole), and anhydrous acetone (300 ml.) were placed into a 500-ml., three-necked flask and the mixture was then refluxed for eighteen hours on a steam bath. After cooling the refluxed mixture, the separated solids were removed by filtration, washed with ether, and allowed to dry. The crude product weighed 128 Gm. (95% of theory) and was used in the subsequent reaction without further purification. Recrystallization from an acetone-ethanol mixture afforded crystals, m. p. 163–164° (decompn.) [reported, m. p. 158–159° (decompn.) (24) and 159° (decompn.) (13)].

**Diethyl Piperidyl-1-acetate-2- $\beta$ -propionate.**—Diethyl pyridinium-1-acetate-2- $\beta$ -propionate bro-

mide (20 Gm., 0.058 mole) was dissolved in water (170 ml.) and glacial acetic acid (30 ml.). The mixture was shaken with platinum oxide (0.1 Gm.) and hydrogen in a standard Parr low pressure hydrogenation apparatus. After the required amount of hydrogen had been absorbed, the mixture was filtered and the filtrate concentrated under partially reduced pressure. The resulting residue was basified with a saturated solution of sodium carbonate and immediately extracted with ether (three 125-ml. portions). The combined ether extract was dried over anhydrous sodium sulfate. After removal of the ether by distillation, the residue was fractionated under reduced pressure to yield 13.5 Gm. (86%) of a colorless liquid, b. p. 131–135°/0.9 mm.,  $n_D^{25}$  1.4634 [reported, b. p. 138–140°/1 mm. (13) and 112–113°/0.35 mm.,  $n_D^{25}$  1.4615 (24)].

**3-Ketoquinolizidine.**—The Dieckmann cyclization of diethyl piperidyl-1-acetate-2- $\beta$ -propionate was performed in a manner almost identical to that described for the preparation of 1-ketoquinolizidine (*loc cit*). A 57% yield of the aminoketone was realized, b. p. 63–65°/0.25 mm.,  $n_D^{25}$  1.4910 [reported, b. p. 62–63°/0.65 mm.,  $n_D^{25}$  1.4926 (24)]. The picate was prepared in ether and recrystallized from acetone at a low temperature, m. p. 180–181° (decompn.) [reported 180–182° (decompn.) (24)].

**3-Hydroxyquinolizidine.**—Reduction of 3-ketoquinolizidine with sodium borohydride in the manner described for 1-hydroxyquinolizidine (*loc cit*) yielded 3-hydroxyquinolizidine in 89% yield, b. p. 83–86°/0.3 mm.,  $n_D^{25}$  1.5020 [reported, b. p. 128°/14 mm. (20)]. The picate was prepared in ether and recrystallized from ethanol, m. p. 161.5–163° [reported, m. p. 161.5–162.5° (20)].

Hydrogenation of 3-ketoquinolizidine catalytically with a platinum oxide catalyst and ferrous sulfate promoter yielded a mixture of the expected 3-hydroxyquinolizidine (61% yield) and quinolizidine, b. p. 41–45°/0.6 mm.,  $n_D^{25}$  1.4830 [reported (25), b. p. 84°/21 mm.,  $n_D^{25}$  1.4796]. The picate was prepared, m. p. 196.5–199° [reported, m. p. 198–199° (25)]. The melting point of this picate was not depressed by admixture with an authentic sample of quinolizidine picate kindly supplied by Dr. V. Boeckelheide.<sup>7</sup>

## Synthesis of Esters<sup>8</sup>

**Method 1 a.—Quinolizidin-3-yl Diphenylacetate Hydrochloride.**—The acid chloride prepared from diphenylacetic acid (3.0 Gm., 0.014 mole) and thionyl chloride (10 ml.) was dissolved in anhydrous benzene (20 ml.) and slowly added with stirring to a mixture of 3-hydroxyquinolizidine (2.0 Gm., 0.013 mole) and triethylamine (2 ml.) dissolved in anhydrous benzene (20 ml.). The reaction mixture was kept cool throughout the addition period. After allowing the reaction to proceed at room temperature for one-half hour the mixture was

<sup>7</sup> Department of Chemistry, University of Rochester, Rochester, N. Y.

<sup>8</sup> See Tables I, II, and III for physical constants and analytical data. Because the syntheses of the quinolizidine esters by three general methods previously reported (13, 24, 25) were treated individually and only a few examples are given, each general category is given. The example given is typical of all syntheses in that group.

<sup>6</sup> The use of absolute ethanol and dry hydrogen chloride gas in the ethanolytic procedure resulted in a 58% yield.

TABLE I.—ESTERS OF 1-HYDROXYQUINOLIZIDINE

R	Method of Prepn	Recrystallized from	M p, °C	Empirical Formulas	Analyses			
					Carbon, %		Hydrogen, %	
	1a				Calcd	Found	Calcd	Found
		Methylene chloride, ethyl acetate, ether	161-163	$C_{21}H_{27}NO_2 \cdot HCl$	71.58	71.47	7.31	7.29
		Isopropanol, acetone, ether	234-236 (decompn)	$C_{23}H_{27}NO_2 \cdot CH_3Br$	64.86	64.19	6.81	6.86
	1a	Methylene chloride, ethyl acetate	210-212	$C_{27}H_{29}NO_3 \cdot HCl$	69.07	68.79	6.55	6.48
		Isopropanol, acetone, ether	225-226	$C_{27}H_{29}NO_3 \cdot CH_3Br$	62.89	62.67	6.16	5.97
	1b, 2	Isopropanol, isopropyl ether	205.5-206.5	$C_{27}H_{29}NO_3 \cdot HCl$	59.14	59.24	7.31	7.30
	2	Ethanol, isopropyl ether, acetone	189-191	$C_{26}H_{27}NO_2 \cdot HCl$	64.96	65.16	7.50	7.46
	3	Ethanol, isopropyl ether	213-215	$C_{23}H_{27}N_2O_2 \cdot HCl$	61.83	61.88	7.46	7.33

TABLE II.—ESTERS OF 2-HYDROXYQUINOLIZIDINE

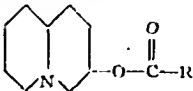
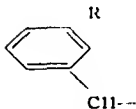
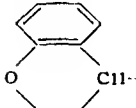
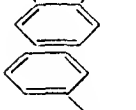
R	Method of Prepn	Recrystallized from	M p, °C	Empirical Formulas	Analyses			
					Carbon, %		Hydrogen, %	
	2				Calcd	Found	Calcd	Found
		Isopropanol, isopropyl ether	242-243 (decompn)	$C_{27}H_{29}NO_3 \cdot HCl$	59.14	59.32	7.31	7.35
	2	Ethanol, isopropyl ether	265-266 (subl)	$C_{26}H_{27}NO_2$	64.96	65.17	7.50	7.54
	3	Ethanol, isopropyl ether, acetone	212.5-214.5	$C_{23}H_{27}N_2O_2$	61.83	61.60	7.46	7.56

heated at 65 to 70° for two hours under a stream of dry nitrogen gas. After cooling, the precipitated triethylamine hydrochloride was removed by filtration and the residue washed with anhydrous benzene (20 ml.) The combined benzene filtrate and washings were concentrated under moderately reduced pressure and the residue dissolved in absolute ethanol. Ethereal hydrogen chloride was added to the ethanolic solution until acid to p-

Hydron paper and the solvents removed under reduced pressure. The resulting glass was hardened by triturating with anhydrous ether and recrystallized from a mixture of methylene chloride and ethyl acetate, m p. 189-190°, yield 4.6 Gm. (92% of theory).

*Quinolizidin-3-yl Diphenylacetate Methobromide.*—This salt was prepared by basifying a solution of the above hydrochloride with ice cold potassium carbo-

TABLE III.—ESTERS OF 3-HYDROXYQUINOLIZIDINE

						Analyses			
		Method of Prepn	Recrystallized from	M p °C	Empirical Formulas	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
		1a	Methylene chloride, ethyl acetate	189-190	$C_{11}H_{11}NO_2 \cdot HCl$	71.58	71.69	7.31	7.22
			Isopropanol, ethanol, ether	213.5-214.5 (decompn)	$C_{11}H_{11}NO_2 \cdot C_3H_7Br$	61.86	61.81	6.81	6.83
		1a	Methylene chloride, ethyl acetate	211-215.5	$C_{11}H_{11}NO_2 \cdot HCl$	69.07	69.15	6.55	6.79
			Ethanol, methanol	270-271 (decompn)	$C_{11}H_{11}NO_2 \cdot C_2H_5Br$	62.89	62.62	6.16	5.99
		1a	Methylene chloride, ethyl acetate	187-188	$C_{11}H_{11}NO_2 \cdot HCl$	70.47	70.25	8.48	8.50
		1a	Methylene chloride, ethyl acetate	211-212	$C_{11}H_{11}NO_3 \cdot HCl$	59.11	59.03	7.31	7.22
		1a	Ethanol, isopropanol	255-255.5	$C_{11}H_{11}NO_3 \cdot HCl$	61.96	61.90	7.50	7.47
		3	Ethanol, isopropanol	213.5-211.5	$C_{11}H_{11}N_2O_2 \cdot HCl$	61.83	61.85	7.46	7.39

nate solution (25% w/v). The basic solution was extracted with ether and the ether extracts washed with water. After drying the extracts over anhydrous sodium sulfate, the ether was removed. The residue was dissolved in a small amount of methanol and an ethereal solution of methyl bromide added in excess. After allowing the solution to stand at room temperature in a stoppered bottle for two days, the precipitate was removed and recrystallized from a mixture of isopropanol, ethanol, and ether to give white crystals, m. p. 243.5-244.5° (decompn.).

**Method 1 b.—Quinolizidin-1-yl 3,4,5-Trimethoxybenzoate Hydrochloride.**—The acid chloride prepared from 3,4,5-trimethoxybenzoic acid (1.5 Gm., 0.0065 mole) and thionyl chloride (5 ml.) was dissolved in anhydrous benzene (20 ml.) and added to 1-hydroxyquinolizidine (2 Gm., 0.013 mole) dissolved in anhydrous benzene (20 ml.). The mixture was stirred and heated at 70° for eight hours. After cooling the reaction mixture, the aminoalcohol hydrochloride (1.3 Gm.) was removed by filtration and washed with anhydrous benzene. The benzene was removed from the filtrate under partially reduced pressure and the residue dissolved in a mixture of benzene and ether. The solution was washed with water and dried over anhydrous potassium carbonate. Ethereal hydrogen chloride was then added to the dried solution and the

precipitate recrystallized from a mixture of isopropanol and isopropyl ether. This yielded 1.3 Gm. of light brown crystals, m. p. 206-209°. A second crop of crystals that precipitated from the acidulated benzene-ether mixture proved to be 3,4,5-trimethoxybenzoic anhydride, m. p. 160-162° [reported, 160-161° (26)].

**Method 2.—Quinolizidin-2-yl Benzoate Hydrochloride.**—In a 200-ml. three-necked flask equipped with an efficient stirrer, reflux condenser, Dean-Stark water separator, and a drying tube were placed Skellysolve C (40 ml.), 2-hydroxyquinolizidine (2 Gm.), and methyl benzoate (1.8 Gm.). The mixture was stirred and heated to reflux and a catalytic amount of sodium hydride paste was added. The reaction mixture was then refluxed for fifteen hours and allowed to cool. Ether (30 ml.) was added, followed by water (20 ml.), and the mixture stirred. The contents were then transferred to a separatory funnel and the aqueous phase removed and discarded. The ether-Skellysolve C solution was washed with water (four 20-ml. portions) and dried over anhydrous potassium carbonate. The solution was filtered and ethereal hydrogen chloride added to the filtrate. The product was collected on a Büchner funnel and washed with isopropyl ether. After drying, the product weighed 3.2 Gm. (84% of theory) and was readily recrystallized from isopropanol or a mixture

TABLE IV.—ANTISPASMODIC TESTING<sup>a</sup>

Compound	Acetylcholine		Histamine		Barium Chloride		Serotonin	
	Part	Comp	Part	Comp	Part	Comp	Part	Comp
Quinolizidin-1-yl diphenyl acetate hydrochloride	100	500	25	500	250	500	25	500
Quinolizidin-1-yl diphenyl acetate methobromide	25	100	100	.	500		100	.
Quinolizidin-1-yl xanthene-9-carboxylate hydrochloride	25	500	100	500	500		25	500
Quinolizidin-1-yl xanthene-9-carboxylate methobromide		5	500	.	100		500	.
Quinolizidin-3-yl diphenyl acetate hydrochloride	250	.	250	.	250			25
Quinolizidin-3-yl diphenyl acetate methobromide	1	10	25		250		25	.
Quinolizidin-3-yl xanthene-9-carboxylate hydrochloride	25	150	100		250			25
Quinolizidin-3-yl xanthene-9-carboxylate methobromide	5	25	.				25	.
Quinolizidin-3-yl phenylcyclohexylacetate hydrochloride	500	1000	500	.	250		25	1000
Methantheline bromide	5	1	.	.				.
Papaverine hydrochloride			.	.	1000		.	.

<sup>a</sup> See text for explanation of values

TABLE V—LOCAL ANESTHETIC ACTIVITY

Compounds (as Hydrochlorides)	Anesthetic Ratio <sup>a</sup>
Quinolizidin-1-yl diphenyl acetate	1 01
Quinolizidin-1-yl xanthene-9-carboxylate	2 42
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate	0 12
Quinolizidin-1-yl phenylcarbamate	0 54
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate	0 86
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate	0 21
Quinolizidin-1-yl phenylcarbamate	1 72
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate	0 40
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate	2 38
Quinolizidin-3-yl xanthene-9-carboxylate	3 43
Quinolizidin-3-yl 3,4,5-trimethoxybenzoate	0 10
Quinolizidin-3-yl benzoate	0 36
Quinolizidin-3-yl phenylcarbamate	0 32
Quinolizidin-3-yl phenylcyclohexylacetate	0 69
Piperocaine (dl-(2-methylpiperidino)-propyl benzoate)	1 00

<sup>a</sup> See text for explanation of values

of ethanol and isopropyl ether, m p 265–266° (subl)

**Method 3.**—*Quinolizidin-3-yl Phenylcarbamate Hydrochloride*—3-Hydroxyquinolizidine (11 Gm) and phenylisocyanate (1 ml) were placed in an Erlenmeyer flask, protected with a drying tube, and heated on a steam bath for five minutes. The solid which formed upon cooling was washed with anhydrous ether and dissolved in hot ethanol. The solution was allowed to cool and ethereal hydrogen chloride added. The precipitate was recrystallized from absolute ethanol to yield 14 Gm (70% of theory) of product, m p 243.5–244.5°. A mixture of isopropanol and ethanol also provided a suitable recrystallizing medium.

## Pharmacological Testing

**Antispasmodic.**—Aqueous solutions of all materials were employed for this testing. The method consisted of initially standardizing the submaximal contractions of isolated guinea pig ileum to acetylcholine chloride (1  $\gamma$ /100 ml), histamine phosphate (5  $\gamma$ /100 ml), barium chloride (2 mg/ml), and serotonin creatinine sulfate (10  $\gamma$ /100 ml). The test material was then introduced into the bath, and two minutes later the ileum was again challenged with the various spasmogens. The numbers in the table represent the  $\gamma$ /100 ml of test bath for each substance tested necessary to prevent spasm when introduced into the bath two minutes before adding the spasmogen. The values under "Part" represent partial inhibition and "Comp" indicates complete inhibition. The precise value was not determined but lies somewhere in between these two values. The results are recorded in Table IV.

**Local Anesthetic.**—All of the compounds tested were rated against piperocaine. This compound was arbitrarily given a value of 1. The procedure used was to inject the test materials in various dilutions intradermally into the clipped backs of guinea pigs and then to determine by pin pricks the completeness of anesthesia over a period of thirty minutes. By a suitable system of scoring, a threshold anesthetic dose (dilution) of a compound producing an average of five anesthetic responses ( $TAD_5$ ) was determined from a graph plotting dilution on a log scale against the average number of anesthetic responses (failure to twitch or phonate) for thirty minutes. The  $TAD_5$  ratio was compared to that of the standard and furnished the basis for comparison of anesthetic potency. Table V summarizes the results.

TABLE VI.—SPONTANEOUS MOTOR ACTIVITY INHIBITION<sup>a</sup>

Compound	Dose, mg/Kg	Number of Rats	Mean % of Initial Activity	Standard Deviation
Quinolizidin-1-yl 3,4,5-trimethoxybenzoate hydrochloride	10	6	46.8	11.0
Quinolizidin-3-yl 3,4,5-trimethoxybenzoate hydrochloride	50	6	28.3	15.9
Reserpine	10	6	2.1	2.3
Chlorpromazine	1	6	34.6	10.3

<sup>a</sup> See text for further explanation

**Spontaneous Motor Activity.**—The tests were conducted on female albino rats in activity cages known as Actophotometers (Metro Industries). The rats, six to a group, were placed individually in the activity cages and a two-hour count was made to establish control values for spontaneous motor activity. The test compounds, dissolved in normal saline, were administered intraperitoneally and the animals replaced in the activity cages. The two-hour experimental count was begun thirty minutes after injection of the compounds. The results are recorded in Table VI.

## REFERENCES

- (1) Baehrach, W. H., *Am J Digest Diseases*, **3**, 711 (1958).
- (2) Sternbach, L. H., and Kaiser, S., *J Am Chem Soc*, **74**, 2219 (1952).
- (3) Randall, L. O., Benson, W. M., and Stoklo, P. L., *J Pharmacol Exptl Therap*, **104**, 281 (1952).
- (4) Rhodes, H. J., and Some, T. O., *This Journal*, **45**, 716 (1956).
- (5) McElvain, S. M., *J Am Chem Soc*, **49**, 2835 (1927).
- (6) McElvain, S. M., and Carney, T. P., *ibid*, **68**, 2592 (1946).
- (7) Miller, I. M., and Weinberg, M. S., Abstracts of Papers Presented at the Am Chem Soc meeting, Atlantic City, N. J., Sept 16-21, 1956, p 11N.

- (8) *Chem Eng News*, **34**, 1760 (1956).
- (9) Bockelheide, V., and Gall, W. G., *J Am Chem Soc*, **76**, 1832 (1951).
- (10) Leonard, N. J., Hay, A. S., Fulmer, R. W., and Gash, V. W., *ibid*, **77**, 413 (1955).
- (11) Clemo, G. R., and Ramage, G. R., *J Chem Soc*, **1931**, 137.
- (12) Clemo, G. R., Metcalfe, T. P., and Raper, R., *ibid*, **1936**, 1129.
- (13) Clemo, G. R., Morgan, W., and Raper, R., *ibid*, **1935**, 1713.
- (14) Reckhow, W. A., and Tarbell, D. S., *J Am Chem Soc*, **74**, 1961 (1952).
- (15) Bockelheide, V., Linn, W. J., O'Grady, P., and Lamborg, M., *ibid*, **75**, 3213 (1953).
- (16) Biel, J. H., Sprengler, E. P., Leiser, H. A., Horner, J., Drukker, A., and Friedman, H. L., *ibid*, **77**, 2250 (1955).
- (17) Reimer, M., and Downes, H. R., *ibid*, **43**, 945 (1921).
- (18) Singer, A. A., and McElvain, S. M., *ibid*, **57**, 1135 (1935).
- (19) Prill, E. A., and McElvain, S. M., *ibid*, **55**, 1233 (1933).
- (20) Leonard, N. J., Swamm, S., Jr., and Figueras, J., Jr., *ibid*, **74**, 1622 (1952).
- (21) Walter, L. A., Hunt, W. H., and Fosbinder, R. J., *ibid*, **62**, 2163 (1917).
- (22) Doering, W. E., and Weil, R. A. N., *ibid*, **69**, 2463 (1917).
- (23) Tullock, C. W., and McElvain, S. M., *ibid*, **61**, 961 (1939).
- (24) Leonard, N. J., and Pines, S. H., *ibid*, **72**, 4931 (1950).
- (25) Bockelheide, V., and Rothchild, S., *ibid*, **69**, 3149 (1917).
- (26) Sharp, T. M., *J Chem Soc*, **1936**, 1235.

# A Study of Certain Analgetic-Antipyretic Compounds\*

By LAWRENCE C. WEAVER and BENEDICT E. ABREU

Two new compounds,  $\alpha$ -phenyl- $\alpha$ -(2-piperidino-ethyl)- $\beta$ -ethyl-butyric acid nitrile and 1-phenyl-2,3-dimethyl-4-(phenylmethyl-morpholino)-methyl-pyrazolone-(5), have been studied by various procedures. These compounds showed no analgetic activity by a thermal method in mice, weak analgetic activity as indicated by blockade of irritant-induced writhing in mice, good antipyretic activity in rats, and no antigranuloma-tous activity in rats.

THE DISCOVERY of therapeutically useful compounds which influence acute or chronic inflammatory conditions, and possess a strong analgetic effect as well, is one of the main goals of pharmacologic research. Analgetic and antiphlogistic activity has been reported for 1-phenyl-2,3-dimethyl-4-(phenyl-methyl-morpholino)-methyl-pyrazolone-(5)<sup>1</sup> by Hengen and co-

workers (1), and for  $\alpha$ -phenyl- $\alpha$ -(2-piperidino-ethyl)- $\beta$ -ethyl-butyric acid nitrile<sup>1</sup> by Kasperek and Pfoepfler (2). These compounds have been compared with several established compounds and the results are presented herein.

## METHODS

The compound of Hengen (1) was administered in the form of its salts, e. g., as the hydrochloride (R-445-H) or as the gentisic acid salt (R-445-G); the compound of Kasperek (2) was used as the hydrochloride (R-154). These code designations will be used throughout this report. Other compounds tested are shown in the results. Male albino mice of the Swiss-Webster strain, male albino rats of the Harlan-Wistar strain, and adult mongrel dogs, unselected as to sex, were used in these studies. Animals had access to feed and water except during the period of testing. All drugs were given either as aqueous solutions or as suspensions in 1% Hercules gum<sup>2</sup> solution.

**Analgetic Experiments.**—A thermal method was used for determining analgetic activity in mice (3). All drugs for analgetic testing were administered intragastrically (i. g.) except for morphine sulfate

\* Received August 21, 1959, from the Department of Pharmacology, Research Center, Pitman Moore Co., Indianapolis, Ind.

Presented to the Scientific Section, A. P. A., Cincinnati meeting, August 1959.

We are indebted to Luise Schoene and Jessa Tempke for technical assistance, and to Dr. Elva G. Shipley, Director, The Endocrine Laboratories, Madison 1, Wis., for antigranuloma testing.

<sup>1</sup> Supplied by Dr. H. C. Stark, Ravensberg G. M. B. A., Konstanz, Germany.

<sup>2</sup> Cellulose gum—CMC—120 high viscosity, Hercules Powder Co.

and normorphine hydrochloride which were given by the intraperitoneal (i. p.) route. Controls of distilled water (i. p.) and morphine were always run simultaneously with test compounds. Six animals were used at each dosage level tested. The ability of drugs to block hydrochloric acid-induced writhing in mice was also determined (4, 5). Simultaneous controls with hydrochloric acid are considered necessary for this procedure.

**Anti-inflammatory Experiments.**—Tests for anti-granulomatous activity were performed in 50-day-old male rats (6). The drugs were administered i. g. daily for seven days to rats previously implanted with four cotton pellets each. Autopsy was performed on the day following the last day of administration, the pellets were removed, extraneous tissue trimmed off, and pellets dried and weighed. The amount of granulation tissue formed was taken as the difference between the initial and final weight of the dried pellet. Drug effect on rat body weight as well as on granuloma weights was measured.

**Antipyretic Experiments.**—The effect of compounds given i. g. on normal body temperature and yeast-induced fever (7) was evaluated in rats. For temperature determinations, thermistor probes<sup>3</sup> were inserted rectally into male rats confined in wire mesh tubes. Two control temperature readings at thirty-minute intervals were made prior to drug injection and served as controls for each group of four animals; saline controls were run simultaneously. Temperature readings were made at hourly intervals. For experiments using fevered rats, control readings were taken prior to yeast injection (3 ec. of a 15% brewers yeast suspension in saline subcutaneously) and again prior to drug administration. The temperature of the room was maintained at  $24 \pm 1^\circ$ .

**Additional Experiments.**—The ability of these compounds to block supramaximal electroshock seizures (M. E. S. test) and metrazol threshold seizures (Met. test) was determined in mice (8). Acute toxicity in mice was determined by the i. g. route. Groups of 10 mice were used at each dosage level in the determination of the  $LD_{50}$ . The  $LD_{50}$  and 95% confidence limits were determined by the method of Litchfield and Wilcoxon (9). Limited toxicity data were obtained in dogs following i. v. and i. g. administration.

## RESULTS

The results of analgetic study in mice are presented in Fig. 1 and in Table I. Several compounds in nontoxic doses caused a significant ( $P < 0.05$ ) lengthening of the reaction time of mice to a thermal stimulus. These compounds in order of potency were morphine > dihydrocodeinone > normorphine. Methylmelubrin, aminopyrine, R-445, and R-154 produced no significant increase. These last two compounds failed to alter significantly the analgetic activity of morphine sulfate.

Several compounds blocked irritant-induced writhing in mice (Table II); acetylsalicylic acid was the most potent. Both of the new compounds showed weak activity although some toxicity was observed at the more effective doses.

Phenylbutazone,<sup>4</sup> but not R-445 or R-154, reduced

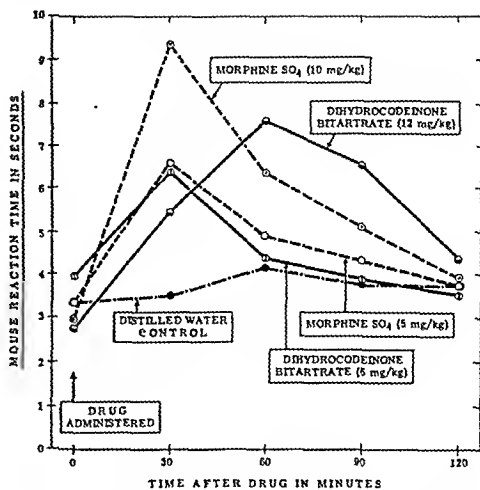


Fig. 1.—Analgetic activity of compounds in mice. All agents produced reaction times significantly greater ( $P < 0.05$ ) than in the controls. The curves for control and morphine represent an average of five experiments.

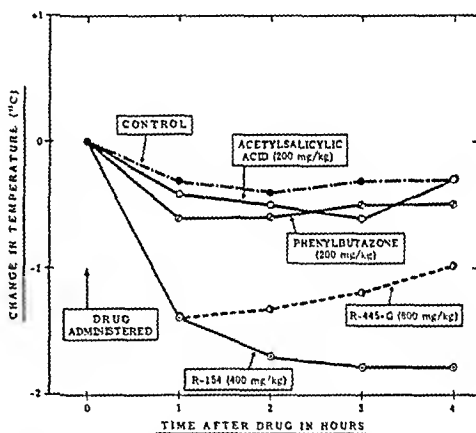


Fig. 2.—Effect of compounds on the normal body temperature of rats. Each point represents the average change in rectal temperature of eight rats.

the granuloma weight in rats (Table III). In fact, the latter two compounds produced a considerable increase in granuloma weight.

Both test compounds produced a considerable decrease in normal body temperature of rats (Fig. 2); in additional studies at one-half these doses, only R-154 produced a significant lowering of normal body temperature. Phenylbutazone and acetylsalicylic acid were not effective at the doses tested. Both test compounds and phenylbutazone were effective in lowering the rectal temperature of rats fevered with yeast (Fig. 3).

R-154 in doses of 100 and 200 mg./Kg. i. g. showed no anticonvulsant effect. R-445-H and R-445-G were ineffective against metrazol threshold seizures; both compounds showed some protection at 200 mg./Kg. i. g. against supramaximal electroshock seizures.

<sup>3</sup> Yellow Springs Instrument Co.

<sup>4</sup> Phenylbutazone (Butazolidin) was supplied by Dr. J. Marrus, Geigy Pharmaceuticals, Yonkers, N. Y.



TABLE I — ANALGESIC ACTIVITY IN MICE AS DETERMINED BY A THERMAL METHOD

Compound	Dose mg /Kg	Response Time of mice, sec				
		0	30	60	90	120
Control		3 76	3 83	3 37	3 49	4 10
Morphine SO <sub>4</sub>	10	4 35	9 75	6 79	5 34	4 39
Methylmelubrin	300	3 53	4 52	4 83	4 03	4 30
Annopyrine	100	3 66	4 32	4 29	4 33	3 70
Control		3 35	3 45	4 89	3 93	3 48
Morphine SO <sub>4</sub>	10	2 63	10 15	6 47	5 29	3 80
R-154	100	3 21	4 03	3 53	4 18	3 57
R-445-H	50	4 21	3 75	4 08	4 55	4 53
Control		3 01	2 93	3 20	3 09	3 78
Morphine SO <sub>4</sub>	10	3 91	8 91	6 53	4 61	3 88
Morphine SO <sub>4</sub>	5	3 43	5 53	5 10	4 53	4 13
Normorphine HCl	10	3 35	5 25	6 33	5 19	4 43
R-154	50	3 48	3 96	4 01	4 33	3 11
R-445-G	100	3 75	3 61	4 53	3 46	3 96
Control		3 50	3 80	3 87	3 98	3 92
Morphine SO <sub>4</sub>	10	3 75	10 46	6 63	5 73	4 14
Morphine SO <sub>4</sub>	5	3 32	5 18	1 43	4 20	3 43
Morphine SO <sub>4</sub>	5					
R-154	100	3 91	5 10	3 75	4 27	4 04
Morphine SO <sub>4</sub>	5					
R-445-H	50	3 17	1 80	4 13	3 88	3 04
Morphine SO <sub>4</sub>	5					
R-445-G	100	3 62	3 62	4 13	3 00	3 31

TABLE II — BLOCKADE OF IRRITANT-INDUCED WRITHING

Drug	Dose mg /Kg	Route	Challenging Time min			Remarks
			30	60	120	
HCl control		i p	9/10 <sup>a</sup>	15/20		
Phenylbutazone	100	i g		9/10		
Phenylbutazone	200	i g		8/10		
R-445-H	50	i g		7/10		
R-445-H	100	i g		4/8	4/10	2 Mice died at 1 hour
R-445-G	50	i g		10/20	3/10	Depression at 2 hours
R-154	50	i g		9/10		
R-154	100	i g	9/10	6/10	6/10	Depression at 1 and 2 hours
HCl control	10	i p		15/20		
Acetylsalicylic acid	100	i g	3/5			
Acetylsalicylic acid	200	i g	0/5			
Acetylsalicylic acid	400	i g	0/10			
Antipyrine	200	s c	6/10			Marked depression
Salicylamide	200	i g	4/5			
Salicylate Na	200	s c	5/5			

<sup>a</sup> Number of mice writhing/number tested

TABLE III — DRUG EFFECT ON GRANULOMA WEIGHTS IN RATS

Treatment	Dose mg	Body Weights Gm		Granuloma Weights		% Difference
		Initial	Final	Wet, mg	Dry, mg	
Controls		170	210	76 6	9 7	
Phenylbutazone	150	176	209	70 2	7 1	-26 8
R-445-H	125	174	204	86 5	+12 9	+23 7
R-445-G	125	175	206	83 5	+ 9 0	+12 3
R-154	150	174	206	85 6	+11 0	+19 6

However, these latter doses were within the toxic range.

The i g LD<sub>50</sub>s (mg /Kg ) and 95% confidence limits in mice were R-154, 700 (636 3-770 0), R-445-H, 260 (203 1-332 8), and R-445-G, 300 (270 3-333 0). Toxic signs were those previously reported (1, 2). In one dog, R-154 at 16 mg /Kg i v. produced apprehension, clonic convulsions, and ataxia, duration of toxic signs was about thirty

minutes. Thirty-two mg /Kg i v (3 dogs) within ninety seconds produced opisthotonos and clonic convulsions lasting about five minutes, followed by occasional jerking, shaking, and ataxia for about one hour. The dogs then became sleepy and arousal was quite difficult, this condition was maintained for more than one hour. At 64 mg /Kg i v (6 dogs) a tonic extensor convulsion occurred followed by the above-mentioned signs. Three dogs died

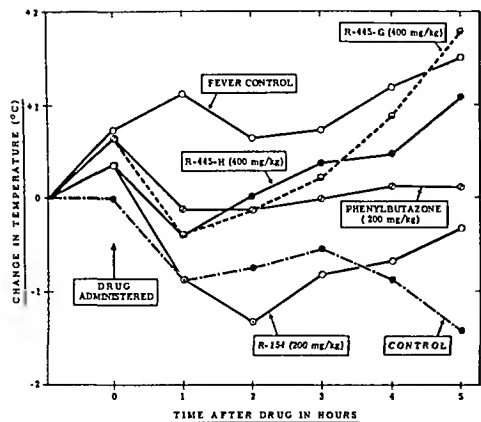


Fig. 3.—Antipyretic activity in rats. Each point represents the average change in rectal temperature of four rats.

within three minutes. At 96 mg./Kg. i. v. (1 dog) death occurred during a tonic extensor convulsion. R-445-H at 150 mg./Kg. i. g. produced emesis in dogs within ten minutes with no other toxic signs. R-445-G, 64 mg./Kg. i. g. (1 dog) produced only retching, and at 16 and 32 mg./Kg. i. p. (1 dog each) produced no toxicity. However, at 64 mg./Kg. i. p. (1 dog) emesis, clonic convulsions, opisthotonos, rigidity in front legs, involuntary jerks, and horizontally maintained ears were observed; toxic signs lasted about one hour.

## DISCUSSION

Hengen and co-workers (1) reported analgetic action for R-445, aminopyrine, phenacetin, and salicylic acid amide, utilizing the analgetic test method of Wolff-Hardy. These investigators found R-445 to be two to three times as potent as aminopyrine. Similarly, Kasperek and Pfroeffer (2) found R-154 to be two to three times more analgetic than aminopyrine when evaluated by the method of electrical stimulation at the roots of the tails of mice. Furthermore, these latter investigators (2) showed that R-154 potentiated morphine analgesia. In the present studies, no significant analgetic effect was noted for aminopyrine, R-445, or R-154 by the thermal method. Furthermore, R-154 or R-445 failed to enhance the analgetic properties of morphine. It would appear that the differences might well be explained by the relative sensitivities of the various analgetic test methods used. The method used here appears satisfactory for analgetics more potent than aminopyrine or acetylsalicylic acid. We have also found acetylsalicylic acid to be quite ineffective when this thermal method for analgetic testing is used. Additional evidence to support the explanation of differences of results may be inferred by the results obtained in the writhing experiment. The well-known weaker analgetics, R-154, R-445, and phenylbutazone all showed some activity; acetylsalicylic acid was the best. Eckhardt (5) and others have observed similar effects as well as activity for the "morphine-type" analgetic. The

fact that R-154 failed to alter electroshock seizure patterns of mice at a dose twice that used in the analgetic studies eliminates threshold elevation as a possible explanation of the analgetic seen.

Neither R-445 nor R-154 was effective in reducing granuloma weights in the cotton pellet implantation method in rats. On the contrary, they produced an increase in granuloma weight comparable in amount to the decrease produced by the effective compound phenylbutazone. Hengen, *et al.* (1), compared the ability of R-445 and phenylbutazone to prevent edema produced by dextran injection into the rat paw. These investigators found R-445 to be twice as effective as phenylbutazone by this method. The reason for the increase in the granuloma weights in our experiments has not been elucidated. However, similar effects have been reported for Compound S (10).

Both compounds proved to be quite effective in controlling fever as well as in lowering normal body temperature.

Considering published results and the data presented herein, it would appear that R-154 and R-445 possess (a) weak analgetic activity, (b) good antipyretic activity, and (c) possibly anti-inflammatory activity depending on the test used. Furthermore, the results suggest that any one test may not be sufficient for evaluating analgetic and anti-inflammatory properties.

## SUMMARY

1 - Phenyl - 2,3 - dimethyl - 4 - (phenyl-methyl-morpholino) - methyl - pyrazolone - (5) and  $\alpha$  - phenyl -  $\alpha$  - (2 - piperidino - ethyl) -  $\beta$  - ethyl - butyric acid nitrile have been compared with known compounds with the following results:

1. No analgetic activity using a thermal method in mice; no enhancement of morphine analgesia; analgetic activity as indicated by blockade of irritant-induced writhing in mice.
2. No antigranulomatous activity in rats.
3. Good antipyretic activity in fevered rats; lowering of body temperature in normal rats.

A possible explanation is presented for the lack of correlation of these data with previously published results.

## REFERENCES

- (1) Hengen, O., Seimer, H., and Doppstadt, A., *Arzneimittel-Forsch.*, **8**, 421(1958).
- (2) Kasperek, H., and Pfroeffer, K., *ibid.*, **8**, 673(1958).
- (3) Chen, J. Y. P., and Beckman, H., *Science*, **113**, 631(1951).
- (4) Vander Wende, C., and Margolin, S., *Federation Proc.*, **15**, 494(1956).
- (5) Eckhardt, E. T., Cheplovitz, F., Lipo, M., and Govier W. M., *Proc. Soc. Exptl. Biol. Med.*, **98**, 186(1958).
- (6) Meier, R., Schuler, W., and Desaulles, P., *Experientia*, **6**, 469(1950).
- (7) Maren, T. H., *J. Pharmacol. Exptl. Therap.*, **101**, 313(1951).
- (8) Swinyard, E. A., Brown, W. C., and Goodman, L. S., *ibid.*, **106**, 319(1952).
- (9) Litchfield, J. T., Jr., and Wilcoxon, F., *ibid.*, **96**, 99(1949).
- (10) Rindani, T. H., *Proc. Soc. Exptl. Biol. Med.*, **37**, 345(1954).

# Paper Chromatography of Some Certified Dyes\*

By F. J. BANDELIN† and J. V. TUSCHHOFF‡

Paper chromatography utilizing a single solvent system may be used to separate and identify the majority of a selected group of 20 commonly used certified dyes. Several of these dyes have similar  $R_f$  values and require other solvent systems. Color reactions for the identification and corroboration of the various dye spots are given and methods for their elution from the paper strip and quantitative determination are discussed. The method, where applicable, is rapid, requiring four to six hours for development of the chromatogram. Five- to fifty-microgram quantities of dyes can be separated and spots containing as little as 5 mcg. of dye can be readily detected with the eye.

THE USE OF certified dyes as coloring agents in pharmaceutical preparations often presents problems of separation, isolation, and identification in the analytical control laboratory. Dyes are used either alone or in mixtures to produce a variety of lines and shades, not only for esthetic appeal in products, but also for purposes of identification and differentiation in liquid, solid, and semisolid products, in solutions, syrups, ointments, creams, tablets, and capsules.

Of the 117<sup>1</sup> colorants on the certifiable lists of the United States, 53 are pigments and 63 are water- or solvent-soluble dyes. Since the separation of all dyes is of more theoretical than practical interest we have limited our investigation to 20 water-soluble dyes most commonly used in pharmaceutical formulations. This list of dyes corresponds reasonably well with the inventory of certified dyes for pharmaceuticals recommended by Peacock(1).

Tilden (2, 3) has reported on the paper chromatography of dyes using multiple solvents for developing the chromatograms of various dyes. We have experimented with various solvent systems attempting to develop a single solvent which might be applied as a rapid, simplified method for the separation and identification of the 20 dyes considered in this investigation. Methods of isolation and identification are also given.

## EXPERIMENTAL

**Materials Required.**—Filter paper, Whatman No. 1 for paper chromatography, 45 cm. X 3 cm. strips.

**Developing Solution.**—An aqueous solution prepared by adding 2 ml. of 28% ammonium hydroxide and 2 ml. of isobutanol to sufficient distilled water to make 100 ml. Amyl alcohol C. P.

**Dye Solutions.**—One per cent solutions of the

following certified dyes: FD&C Orange No. 1, D&C Orange No. 3, D&C Orange No. 4, FD&C Yellow No. 1, FD&C Yellow No. 5, FD&C Yellow No. 6, FD&C Green No. 1, FD&C Green No. 2, FD&C Green No. 3, D&C Green No. 5, FD&C Blue No. 1, FD&C Blue No. 2, D&C Blue No. 4, FD&C Red No. 1, FD&C Red No. 2, FD&C Red No. 3, FD&C Red No. 4, D&C Black No. 1, D&C Brown No. 1, FD&C Violet No. 1.

**Procedure.**—To determine the  $R_f$  values of the individual dyes under consideration, the standard dye solution is spotted on the starting line, a point 13 cm. from the lower end of the paper strip. At the midpoint on this line, a spot of dye solution, about 0.5 cm. in diameter is applied by means of a glass capillary containing the solution.

The solution is transferred to the paper by merely touching the end of the capillary and is absorbed into the paper. About 8 cm. below the starting line two slits, 1 cm. apart and parallel to the major axis, are cut in the paper. These slits are of sufficient length to form a loop in which may be inserted a piece of glass rod 3 cm. in length. Thus weighted, the strips are suspended in a large glass jar either by fastening to the under side of the cover with Scotch tape or any other appropriate method so that the strips hang perpendicularly without touching the sides of the jar or each other, and so that the weighted end is immersed to the extent of about 3 cm. into the solvent which travels vertically, by capillary action, through the paper to form ascending chromatograms. The jar is tightly closed and sealed, and after a sufficient length of time has elapsed (usually four to six hours) to permit the development of the chromatogram, the strips are removed, the solvent front marked with a pencil, and the strip air-dried. The various dye spots are noted and the  $R_f$  values calculated as follows:

$$R_f = \frac{\text{Distance traversed by the dye zone}}{\text{Distance traversed by the solvent front}}$$

## APPLICATION

In pharmaceutical preparations where a mixture of dyes is suspected, these dyes may be extracted from aqueous solutions with *n*-amyl alcohol after acidifying the solution with hydrochloric acid. The following procedure may be used for the determination of the individual dyes in a dye mixture used for sugar-coated tablets.

\* Received August 21, 1959, from the Research Laboratories of Flint, Eaton & Co., Decatur, Ill.

† Present Address: Strong Cobb Arner Inc., Cleveland, Ohio.

‡ Present Address: A. E. Staley Co., Decatur Ill. Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

<sup>1</sup> Such dyes as have been decertified or changed in certification status retain their original designation in this work.

**Method for Sugar-Coated Tablets.**—A number of sugar-coated tablets, usually from 5 to 20, depending upon the size, are placed in a 125-ml Erlenmeyer flask containing 25 ml of water. The contents are swirled with a rotary motion until all of the colored sugar coating is dissolved. The solution is then decanted from the tablets into a 50 ml volumetric flask through a small funnel with Whatman No. 1 filter paper. The volume is made up to 50 ml by the addition of water through the filter. The solution or a suitable aliquot is transferred to a 125 ml separatory funnel and 2 ml of concentrated hydrochloric acid added for every 5 ml of solution. The solution is then extracted with two 5 ml portions of *n*-amyl alcohol. This extracts the dyes and serves to concentrate them in a smaller volume. Should further concentration be required, the amyl alcohol may be extracted with two 2.5 ml portions of 2% ammonium hydroxide solution, then to the combined ammonium hydroxide extracts 2 ml of concentrated hydrochloric acid are added and the dye again extracted, this time with two 1-ml portions of amyl alcohol.

Using the technique previously described the amyl alcohol solution of the dye(s) is spotted on the paper strip with a capillary, the spot is dried in a warm air blast, and the chromatogram developed with aqueous ammonia-isobutanol solution and air-dried. The colored spots are noted and the  $R_f$  values calculated.

**Compressed Tablets and Powders.**—Dyes contained in compressed tablets or powders may be isolated by extracting the powdered tablets or the powder with 2% ammonium hydroxide, acidifying with concentrated hydrochloric acid, and continuing the extraction using *n*-amyl alcohol as given under the method for sugar-coated tablets.

**Liquids.**—Dyes contained in aqueous liquid products may be isolated by acidifying the liquid with concentrated hydrochloric acid, 2 ml of acid to 5 ml of solution, and extracting the dye with *n*-amyl alcohol as given under the method for sugar-coated tablets.

## DISCUSSION

As is apparent from Table I, dyes of the same color group frequently have similar  $R_f$  values, thus making separation with the recommended solvent difficult. By allowing a longer time for the chromatogram to develop, thereby having the spots traverse a greater distance, better resolution can be obtained.

FD&C Reds No. 1, 2, and 4 have similar  $R_f$  values but can be separated satisfactorily by allowing ten to twelve hours for development of the chromatogram. This longer time produces better resolution with discreet spots which, although close together, are readily discernible and identified.

FD&C Blue No. 1 and FD&C Green No. 2 have similar  $R_f$  values. These may be separated using a solvent system composed of 80 parts of phenol and 20 parts of water and allowing to develop for twelve hours.

FD&C Yellow No. 1 and FD&C Red No. 4 may be separated using a solvent composed of 100 parts of *n*-butanol, 100 parts of 2% ammonium hydroxide, and 50 parts of ethanol. This solvent gave  $R_f$  values of 0.44 and 0.20, respectively, for these dyes.

TABLE I.— $R_f$  VALUES OF CERTIFIED DYES BY PAPER STRIP CHROMATOGRAPHY<sup>a</sup>

Dye	$R_f$ Value
Orange	
FD & C No. 1	0.264
D & C No. 3	0.771
D & C No. 4	0.168
Yellow	
FD & C No. 1	0.465
FD & C No. 5	0.662
FD & C No. 6	0.710
Green	
FD & C No. 1	0.713
FD & C No. 2	0.940
FD & C No. 3	0.959
D & C No. 5	0.615
	0.291
Blue	
FD & C No. 1 <sup>b</sup>	0.932
FD & C No. 2	0.216
D & C No. 4 <sup>b</sup>	0.940
Red	
FD & C No. 1	0.433
FD & C No. 2	0.421
FD & C No. 3	0.102
FD & C No. 4	0.306
Black	
D & C No. 1	0.084
Brown	
D & C No. 1	0.135
Violet	
FD & C No. 1	0.745

<sup>a</sup> Ammonia-isobutanol-water developer.

<sup>b</sup> Sodium and ammonium salts, respectively, of the same base.

There appears to be little relationship between the molecular weight and the migratory characteristics of these dyes. The  $R_f$  values are easily affected by changes in solvent and temperature and to some extent by the amount of dye used. Faster moving dyes show a greater variation in  $R_f$  values than do those with lower  $R_f$  values.

Individual dyes gave discreet spots and good separation. Although relatively good separation could be obtained with 2% aqueous ammonium hydroxide solution alone, the addition of 2% isobutanol gave better resolution and more compact spots. To obtain constant and reproducible  $R_f$  values, conditions must be standardized and very closely controlled. Reference colors should be run in the same jar at the same time, since slight deviations in conditions cause the dye spots to become capricious with resultant wide variation in  $R_f$  values. Typical  $R_f$  values obtained in a number of determinations are given in Table I. These  $R_f$  values are valid for identification only when they are obtained under conditions identical to those under which they were obtained for the reference color. Such factors as the presence of extraneous material, concentration of the dye, and the presence of similar color and  $R_f$  values, all affect  $R_f$  values. As many as eight dyes can be separated successfully on a single chromatogram and identified (see Fig. 1). Separation can usually be carried out in four to six hours. Confirmation of the identity of similar or like colors may be carried out by subjecting the moist strips of paper containing the dye spots to ammonia fumes and/or hydrochloric acid fumes to develop characteristic color reactions given in Table II.

TABLE II.—COLOR REACTION OF VARIOUS DYE SPOTS OF CHROMATOGRAMS TO FUMES OF HYDROGEN CHLORIDE AND AMMONIA

Dye	HCl	NH <sub>4</sub>
Orange		
FD & C No. 1	Violet	Dark Red
D & C No. 3	Orange	Yellow-orange
D & C No. 4	Red	Dark orange
Yellow		
FD & C No. 1	Pale yellow	No change
FD & C No. 5	Darker yellow	No change
FD & C No. 6	Reddish	No change
Green		
FD & C No. 1	Pale Yellowish-orange	Decolorized
FD & C No. 2	Pale yellowish-orange	Decolorized
FD & C No. 3	Orange	Blue
D & C No. 5	Pale green	Pale blue
Blue		
FD & C No. 1	Yellow	No change
FD & C No. 2	Darker green	Bluish-green
FD & C No. 4	Pale yellow	No change
Red		
FD & C No. 1	Slightly paler	No change
FD & C No. 2	Slightly darker	No change
FD & C No. 3	Orange yellow	No change
D & C No. 4	Darker red	Orange-yellow
Black		
D & C No. 1	Bluish-green	Blue
Brown		
D & C No. 1	Reddish-brown	Yellowish-brown
Violet		
FD & C No. 1	Yellow	Decolorized

The most positive identification is by reference to spectrometric curves. For the latter, dyes can be eluted from the paper with 50% ethanol containing 0.5% of sodium acetate after cutting out the section of paper containing the spot, macerating it in a centrifuge tube, centrifuging, and reading the clear, supernatant liquid. Spectrometric curves plotted on solutions can be compared with standard reference curves for these dyes (4).

From 5 to 50 meg. of the dye can be easily separated with the 5-meg. spot producing sufficient color to be detectable by the eye.

SUMMARY

The separation and identification of 20 commonly used certified dyes can be achieved, with few exceptions, through paper chromatography using a single solvent system composed of 2 per cent aqueous ammonium hydroxide solution containing 2 per cent isobutanol. Because of discrepancies of the *R<sub>f</sub>* values obtained for the dyes due to variation of conditions and techniques during development of the chromatogram, *R<sub>f</sub>* values alone cannot be relied upon for positive identification of the dye. For this reason, confirming colorimetric tests and spectrometric

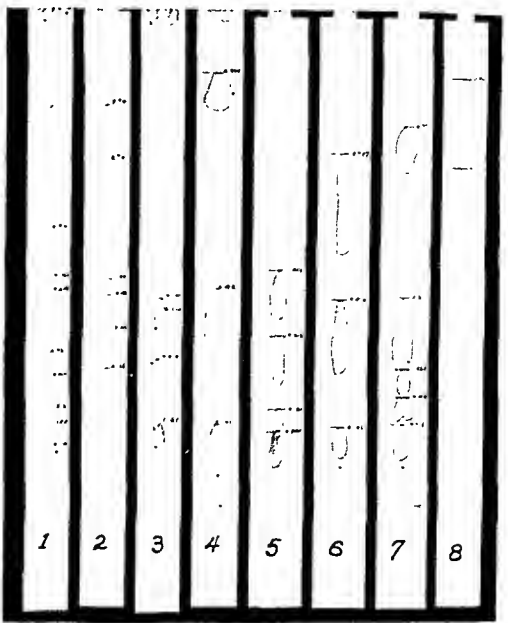


Fig. 1.—Position of spots given by various dyes; reading from the top of the strip down.

- Strip No. 1: FD&C Blue No. 1, D&C Orange No. 3, FD&C Yellow No. 5, FD&C Yellow No. 1, FD&C Red No. 4, FD&C Blue No. 2, D&C Orange No. 4, FD&C Red No. 3.
- Strip No. 2: FD&C Blue No. 1, D&C Orange No. 3, FD&C Yellow No. 5, FD&C Red No. 1, FD&C Red No. 4, D&C Orange No. 4.
- Strip No. 3: D&C Orange No. 3, FD&C Yellow No. 5, FD&C Yellow No. 1, D&C Orange No. 4.
- Strip No. 4: FD&C Blue No. 1, FD&C Red No. 2, FD&C Red No. 3.
- Strip No. 5: FD&C Red No. 1, FD&C Red No. 4, D&C Brown No. 1, D&C Black No. 1.
- Strip No. 6: FD&C Violet No. 1, FD&C Red No. 2, FD&C Red No. 3.
- Strip No. 7: D&C Orange No. 3, FD&C Red No. 2, FD&C Orange No. 1, D&C Orange No. 4, FD&C Red No. 3.
- Strip No. 8: FD&C Green No. 2, FD&C Green No. 1.

reference curves are relied upon for definite identification.

Methods for the extraction and concentration of the dyes from certain pharmaceutical dosage forms are given along with the general method for the chromatography of the dyes.

REFERENCES

(1) Peacock, W. H., *Drug & Cosmetic Ind.*, 1952, March, April, May.

(2) Tilden, D. H., *J. Assoc. Offic. Agr. Chemists*, 35, 423 (1952).

(3) Tilden, D. H., *ibid.*, 36, 810(1953).

(4) Peacock, W. H., "The Application Properties of the Certified Coal Tar Colors," Calco Technical Bulletin #715, American Cyanamid Co.

# Adsorption of Lipid-Soluble Substances by Human Keratin\*

By DALE E. WURSTER and ROBERT E. DEMPSKI

The adsorption of various lipid-soluble alcohols, esters, acids, and mixtures from *n*-heptane solutions on columns composed of powdered, excised, human callous tissue was investigated. Experimental evidence indicates that unsaturated and 2-hydroxy fatty acids were adsorbed. Infrared analysis substantiated the selective adsorption of acids from mixtures composed of human skin lipids and wool fat acids.

MANY LIPID-SOLUBLE SUBSTANCES are commonly applied to the surface of human skin in the form of pharmaceutical and cosmetic preparations. Natural human skin lipids, of course, are also in intimate contact with the keratin layer of skin. Several studies have dealt with the adsorption of various ions by protein (1-4) and the composition of human skin lipids has also been extensively investigated (5, 6). However, little information is available regarding the possible adsorption of the components of skin lipids or other lipid-soluble substances by human keratin.

This investigation was, therefore, initiated in an attempt to determine whether keratin would adsorb certain pure acids, alcohols, and esters and whether selective adsorption would occur from lipid-soluble mixtures such as skin lipids, Lantrol, and wool fat acids.

## EXPERIMENTAL

**Procedure.**—Excised human callous tissue<sup>1</sup> which had not been treated with chemical agents prior to removal was used as a source of keratin. Although certain differences in composition may exist between callous tissue and the normal keratin layer of the skin, callous tissue was the only type that could be obtained in adequate amounts for this study. The keratin was dried for four days over concentrated sulfuric acid and then reduced to a powder in a wedgewood mortar. The powder was fractionated by passing it through standard 100-, 140-, and 200-mesh sieves. The skin lipids were then completely removed from the powder by continuous extraction with anhydrous reagent grade ether for forty-eight hours at room temperature. Since no heat was employed in the extraction procedure, one

pound of ether was used to extract each 2.0-Gm. sample of keratin.

The density of the extracted keratin was found to be 1.19 by the pycnometer method using ether as the displacing fluid. The average particle diameter of the collected fractions was determined with a Fisher sub-sieve sizer (model 14-312) using 0.65, 0.60, and 0.55 porosities.

A known weight of powdered keratin was slurried in purified *n*-heptane and placed in an adsorption column having a 12-mm. diameter and 38-cm. length. Solutions containing various lipid-soluble acids, alcohols, esters, and mixtures in purified *n*-heptane were passed through the columns at the rate of 1 ml. per five minutes. Following this, *n*-heptane was passed through the column until only the pure solvent was obtained.

The amount of adsorbed material was found from the difference between the total amount of material placed on the column and the weight of unadsorbed material. The adsorbed material was eluted from the column with anhydrous ether and subjected to infrared analysis on a Beckman I. R.-5.

In the case where the adsorption columns contained hydrated keratin, the desiccated keratin was first exposed to an atmosphere saturated with water vapor in a closed vessel at room temperature for one to four days. Samples containing 22 to 46% water were thus obtained. Adsorption studies on the hydrated keratin were then carried out according to the previously described procedure. However, it was also necessary to run a blank in this case as a small amount of water-soluble material (0.1-0.3 mg. per 5-ml. fraction), which may have been free amino acids (7), was removed from the column.

## RESULTS AND DISCUSSION

Table I shows the various substances which were tested for adsorption on columns composed of both dehydrated and hydrated keratin. As indicated, of the substances tested, only unsaturated (linoleic and linolenic) and hydroxy (2-hydroxystearic) fatty acids and a fraction of the wool fat acids and skin lipids were adsorbed. In an initial study, oleic acid appeared to adsorb weakly on keratin; however, this could not be substantiated in subsequent tests. None of the lipid-soluble esters, alcohols, or Lantrol components appeared to be adsorbed.

After elution of the adsorbed fatty acids with ether the keratin was again capable of adsorbing these acids. However, particle size measurements on this reprocessed keratin showed an increased average diameter which was probably due to aggregation of the fine powder.

**Linoleic, Linolenic, and 2-Hydroxystearic Acids.**—

In Table II it can be observed that 2-hydroxystearic acid was more strongly bound than linolenic, and the latter was bound more strongly than linoleic. The adsorption of certain aromatic hydroxy ac-

\* Received August 21, 1959, from the School of Pharmacy, University of Wisconsin, Madison.

This study was supported in part by a grant from the Malmstrom Chemical Corp., Newark, N. J. Presented to the Scientific Section, A. Ph. A. Cincinnati meeting, August 1959.

<sup>1</sup> The authors are grateful to the chiropodists of Madison, Wis., especially Dr. R. D. Harmon, and to the Chicago, Ohio, and Illinois Colleges of Chiropody for the callous tissue used in this investigation.

TABLE I—ADSORPTION OF VARIOUS LIPID-SOLUBLE SUBSTANCES ON DEHYDRATED AND HYDRATED KERATIN<sup>a</sup>

Test Substance	Dehydrated Keratin	Hydrated Keratin <sup>b</sup>
Cholesterol	—	—30
Stearyl alcohol	—	—31
Ricinoleyl alcohol	—	—
Cholesteryl palmitate	—	—26
Tristearin	—	—32
Wool fat acids	+	+30
Stearic acid	—	—36
Palmitic acid	—	—28
Oleic acid	—	—22
Linoleic acid	+	+25
Linolenic acid	+	+35
2-Hydroxystearic acid	+	+30
Lanolin	—	—46
Human skin lipids	+	+27

<sup>a</sup> +, adsorption, —, no adsorption<sup>b</sup> Per cent water present

on wool protein was previously reported by Bradley and Easty (3) but no mention of the adsorption of the above fatty acids on human keratin was found in the literature

**Hydrated Keratin.**—Since human keratin normally contains water, the effect of hydration of the keratin on adsorption was also studied. In this case 2-hydroxystearic acid which was the most strongly adsorbed acid investigated was employed. Figure 1 is a plot of the mg of acid adsorbed per Gm of keratin (average particle diameter 4.3  $\mu$ ) versus the amount of water per Gm of dry keratin after hydration. From these data it appears that hydration of the keratin causes an increase in the amount of the acid adsorbed. This increase may possibly be explained on the basis of the unfolding of the protein molecule following hydration which may then expose more functional groups.

**Human Skin Lipids.**—The human skin lipids used in this work were obtained by the method described by Tingstad, Wurster, and Hugueni (8). According to Wheatley (5) skin lipids contain 28% free fatty acids consisting mainly of stearic, oleic, and palmitic acids and smaller amounts of linoleic, linolenic, and others.

The adsorption data for human skin lipids are given in Table II. The concentration (46.3 mg/10 ml) used in these determinations represented the

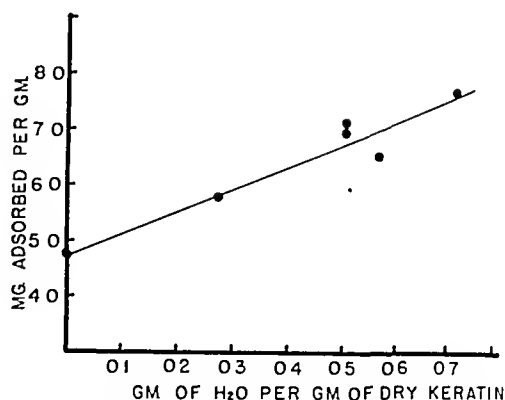


Fig 1—Effect of keratin hydration on the adsorption of 2-hydroxystearic acid

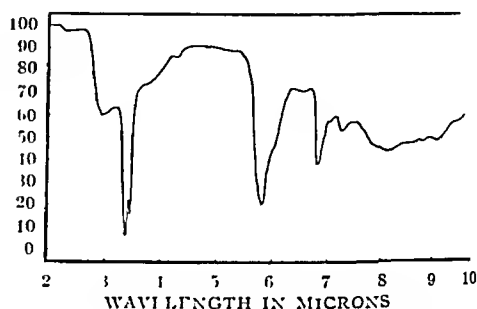


Fig 2—Infrared spectrum of skin lipid adsorbate

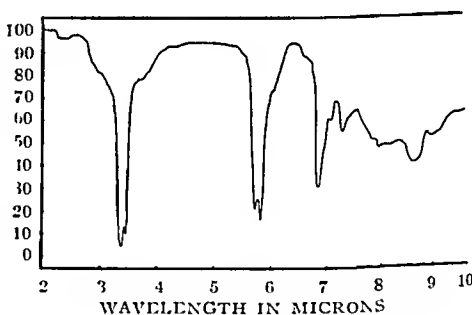


Fig 3—Infrared spectrum of whole skin lipids

TABLE II—ADSORPTION DATA FOR VARIOUS FATTY ACIDS AND HUMAN SKIN LIPIDS ON DEHYDRATED KERATIN

Test Substance	Wt of Keratin Column, Gm	Average Diameter of Keratin, $\mu$	Concn of Heptane Solution	mg Adsorbed per Gm of Keratin
Linoleic acid	8.32	5.05	48.4 mg/10 ml	0.42
	10.29	19.5	48.4 mg/10 ml	0.41
	11.91	22.9	48.4 mg/10 ml	0.36
Linolenic acid	7.81	5.1	42 mg/10 ml	0.56
	9.80	20.1	42 mg/10 ml	0.56
	11.71	23.2	42 mg/10 ml	0.42
2-Hydroxystearic acid	6.92	5.85	8.9 mg/15 ml	0.88
	9.06	23.3	8.9 mg/15 ml	0.66
	11.33	26.8	8.9 mg/15 ml	0.52
Human skin lipids	5.94	6.1	43.3 mg/10 ml	0.37
	7.97	23.7	43.3 mg/10 ml	0.34
	10.61	27.0	43.3 mg/10 ml	0.31

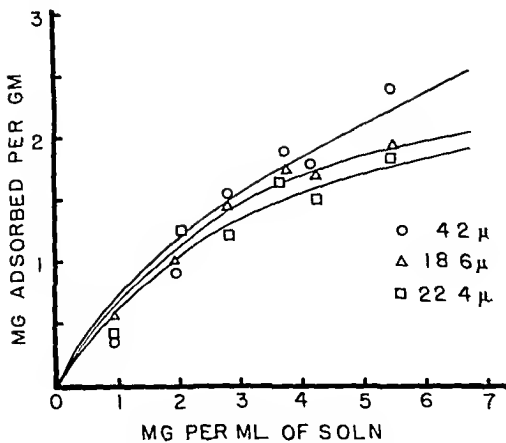


Fig 4.—Effect of solution concentration and adsorbent particle size on the adsorption of wool fat acids

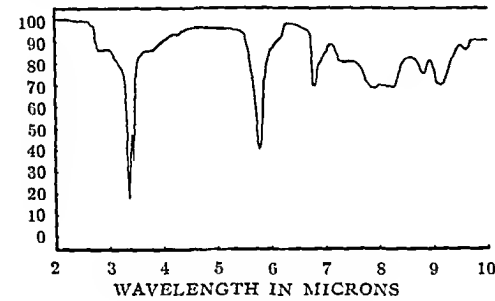


Fig 5—Infrared spectrum of wool fat acids adsorbate

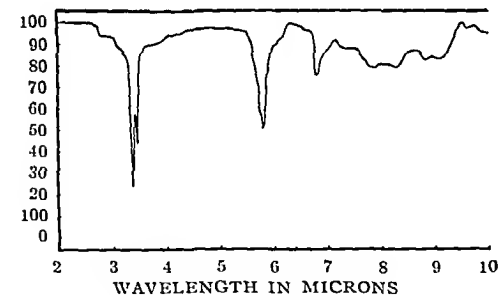


Fig 6—Infrared spectrum of 2-hydroxystearic acid.

*n*-heptane-soluble fraction from 52.9 mg. of whole skin lipids. To confirm the identity of the material adsorbed on the keratin column an infrared spectrum was obtained (Fig 2). For comparison, a spectrum of whole skin lipids is shown in Fig 3. The 5.7  $\mu$  peak is characteristic of esters and the one at 5.8  $\mu$  is characteristic of acids. Thus, the 5.8  $\mu$  peak indicates that the skin lipid adsorbate is composed mainly of acids.

**Wool Fat Acids.**—A mixture of wool fat acids was used to determine both the effect of varying the

concentration of the acids in the *n*-heptane solution and the effect of the particle size of the keratin on the amount of acid adsorbed. According to Weitkamp (9), wool fat contains 32 acidic components. These were classified as normal fatty acids, 2-hydroxy acids, iso acids, and anteiso acids. Horn (10) reported that 2-hydroxy acids represent 27.8% of the wool fat acid mixture. In Table III it can be

TABLE III—ADSORPTION DATA FOR WOOL FAT ACIDS ON DEHYDRATED KERATIN

Concn of <i>n</i> Heptane Solutions, mg /5 ml	Av Diameter of Keratin Powder, $\mu$	mg Adsorbed per Gm of Keratin	% of Total Acids Adsorbed
4.7	4.2	0.35	14.9
9.8	4.2	0.9	18.4
14.0	4.2	1.55	22.2
18.6	4.2	1.9	20.5
20.9	4.2	1.8	17.2
27.2	4.2	2.4	17.6
5.0	18.6	0.55	22.0
9.9	18.6	1.0	20.2
14.1	18.6	1.45	20.6
18.9	18.6	1.75	18.5
21.2	18.6	1.7	16.0
27.4	18.6	1.95	14.2
4.8	22.4	0.4	16.7
10.3	22.4	1.25	24.3
14.1	22.4	1.2	17.0
18.4	22.4	1.65	17.9
21.2	22.4	1.5	14.1
27.4	22.4	1.85	13.5

observed that the keratin adsorbed from 13.5 to 24.3% of the acid mixture placed on the column.

Figure 4 shows that the amount of material adsorbed increased as the concentration of the *n*-heptane solutions increased, and adsorption decreased as the particle size of the adsorbent increased, as would be expected. Colvin (4), working with other adsorbates and adsorbent protein materials, obtained similar plots.

In Fig. 5 it can be observed that the infrared spectrum is almost identical to the spectrum obtained with 2-hydroxystearic acid (Fig. 6). Of particular interest is the absorption in the 2.8  $\mu$  region which represents the hydroxyl group and which is present in both cases.

REFERENCES

(1) Davis, B. D., and Dubos, R. J., *J. Exptl. Med.*, **86**, 215 (1947).  
(2) Klotz, I. M., Trivush, H., and Walker, F. M., *J. Am. Chem. Soc.*, **70**, 2935 (1948).  
(3) Bradley, W., and Easty, G. C., *J. Chem. Soc.*, **1951**, 499.  
(4) Colvin, J. R., *Can. J. Chem.*, **30**, 973 (1952).  
(5) Wheatley, V. R., *Am. Perfumer*, **68**, 37 (1956).  
(6) Weitkamp, A. W., Smiljanic, A. M., and Rothman, S., *J. Am. Chem. Soc.*, **69**, 1936 (1947).  
(7) Schmidly, B., and Paschoud, J. M., *Dermatologica*, **110**, 315 (1955).  
(8) Tingstad, J. E., Wurster, D. E., and Higuchi, T., *THIS JOURNAL*, **47**, 188 (1958).  
(9) Weitkamp, A. W., *J. Am. Chem. Soc.*, **67**, 447 (1945).  
(10) Horn, D. H. S., Hougden, F. W., and von Rudloff, E., *Chem. & Ind.*, **1953**, 106.



# Yellow Phenolphthalein II\*

By MAX H. HUBACHER

Two of the by-products formed in the commercial synthesis of yellow phenolphthalein have been isolated and tentatively characterized. Degradation studies have provided subsequent compounds which have been shown to be identical with authentic samples prepared by direct synthesis. Additional evidence is presented through evaluation of data from infrared spectroscopy.

**YELLOW PHENOLPHTHALEIN** is a widely used laxative, manufactured by condensing phenol with phthalic anhydride. It is of yellow color, contains at least 9.4 per cent phenolphthalein and several other compounds that have been isolated and identified (1).

Two other compounds have also been isolated from yellow phenolphthalein, each in amounts of less than 0.1 per cent, one melting at 250° (V) and the other at 254° (X). Compound V has been briefly described previously (1). Much experimental information has been collected on the two compounds. Even though their structural formulas have not been definitely established, it is felt that the information collected so far should be recorded. Circumstances prevent a continuation of this work.

Compound V,  $C_{31}H_{20}O_6$ , has two lactone groups and one OH-group, and melts at 250°. On reduction of V, a dicarboxylic acid (Vd) is formed. All of this has already been reported in the first paper on yellow phenolphthalein. Now, a monobenzoyl derivative of V has also been made. When the dicarboxylic acid Vd was decarboxylated and the resulting oil distilled with zinc dust, a well-defined compound (VI),  $C_{26}H_{20}O$ , melting at 126°, was obtained. This colorless compound is neither soluble in dilute aqueous alkalis nor in concentrated sulfuric acid. Spectroscopic examination of VI suggests it to be a derivative of 9-phenyl- or 9-phenylxanthene. It was found to be identical with 2-benzyl-9-phenylxanthene, the synthesis of which is described.

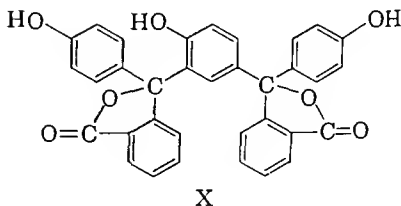
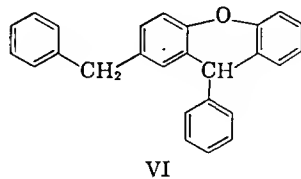
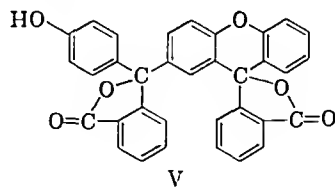
The formation of 2-benzyl-9-phenylxanthene gives support to the formula suggested for V. The carbon skeleton of this xanthene derivative VI can also be detected in V. It is admitted that structural evidence obtained by zinc dust distillation must always be evaluated with caution, but in the phthalide field, this reduction

method has been helpful in structure evaluation (2).

Compound X melts at 252–255°, has the probable empirical formula of  $C_{31}H_{22}O_7$ , and contains one mole more water than V. Neither the acetyl- nor the methyl-ether could be obtained in crystalline form. However, the determination of active hydrogen suggests that X has three OH-groups. The new compound forms solvate crystals with various solvents. The one with two moles of acetic acid was studied especially. Compound X dissolves in dilute aqueous alkalis with the same pink color as phenolphthalein.

On KOH fusion of X, phenol, benzoic acid, and *p*-hydroxybenzoic acid were obtained. The pink color of an alkaline solution of X fades very slowly (3), and *o*-(*p*-hydroxybenzoyl)-benzoic acid could be isolated. When X was subjected to the Dakin reaction, i. e., treatment with alkaline hydrogen peroxide (3), then phthalic acid, hydroquinone, and 3-(*p*-hydroxyphenyl)-3-(*m*,*p*-dihydroxyphenyl)-phthalide (XI), also named phenol-catechol-phthalein, were obtained. The formation of XI is evidence that phenolphthalein is part of the molecule of X, and that it is substituted in *ortho*-position to one of its hydroxyl groups. When X is reduced, a dicarboxylic acid XII is formed, proof that X has two lactone groups.

The following tentative structural formulas are given for V and X:



\* Received June 11, 1950, from the Research Laboratory of Ex-Lax, Inc., Brooklyn 17, N. Y.  
The author wishes to acknowledge the experimental assistance of Douglas Curtin.

The formula for V suggests that this compound may be synthesized from *o*-(*p*-hydroxybenzoyl)-benzoic acid and fluoran; compound X from the same acid and phenolphthalein. It should be remembered that the *o*-(*p*-hydroxybenzoyl)-benzoic acid is an intermediate in the formation of phenolphthalein from phenol and phthalic anhydride (4).

Some 2-hydroxyanthraquinone could be isolated from yellow phenolphthalein, but only in quantities of less than 0.01 per cent. This small amount of this yellow compound is not responsible for the main yellow color of yellow phenolphthalein. The yellow compound or compounds mainly responsible for the yellow color of yellow phenolphthalein have not been isolated. They seem to be quite elusive.

### EXPERIMENTAL<sup>1</sup>

**Isolation of the compounds.**—The isolation of compounds from yellow phenolphthalein was done essentially as described on page 264 of the first paper (1). The brownish, amorphous residue, which was left after no more crystals could be obtained, was dissolved in absolute ether. The yellowish-brown solution, showing greenish fluorescence, was passed through a Tswett column, 4 × 80 cm. inside dimensions, filled with Florisil (100/200 mesh, Floridin Co., Tallahassee, Florida). The first percolates contained phenolphthalein and isophenolphthalein or 3-(*o*-hydroxyphenyl)-3-(*p*-hydroxyphenyl)phthalide. Then came percolates with compound V, and finally percolates with X in them. The pink layer of the ether-washed column, on extraction with ethanol, usually yielded additional small quantities of X.

Separation on the column is not sharp and sometimes, crystal mixtures of V and X are obtained. Separation of such mixtures is easily effected since only compound X is soluble in 0.1 *N* sodium hydroxide.

**Benzoylderivative of V.**—By warming 0.20 Gm. of V, 0.24 ml. pyridine, and 0.15 ml. benzoylchloride, and recrystallizing the crude benzoylderivative several times from ethanol, very fine needles arranged in warts and melting at 207.1–209.8° were obtained.

*Anal.*—Calcd. for  $C_{21}H_{12}O_7$ : C, 78.34; H, 3.82; one benzoyl 16.7. Found: C, 78.56; H, 4.11; benzoyl 16.8 ± 1.9.

**Compound VI.**—Compound V was reduced to dicarboxylic acid Vd, and the latter decarboxylated to the oily compound, as described on page 265 of the first paper (1).

The oil obtained by decarboxylation of 1.7 Gm. of Vd, was mixed with 5 Gm. of  $ZnCl_2$ , 1.0 Gm. NaCl, and 3.0 Gm. of zinc dust (5) and the mixture gradually heated in an atmosphere of hydrogen to 260°. A yellowish, fluorescent oil condensed on the cold part of the tube. This oil slowly crystallized. This new compound, after several crystal-

lizations from ethanol and sublimation at 120° and 10  $\mu$  pressure, formed colorless crystals melting at 125.8–126.5° (VI). It is insoluble in both 1 *N* sodium hydroxide and concentrated sulfuric acid.

*Anal.*—Calcd. for  $C_{26}H_{20}O$ : C, 89.62; H, 5.78 mol. wt. 348. Found: C, 89.36 ± 0.26; H, 5.79 ± 0.14; mol. wt. 351; 316 (Rast).

A mixture of this compound VI with 2-benzyl-9-phenylxanthene (m. p. 126.0–126.5°) melted at 126.0–126.6°.

**Compound X.**—The compound crystallizes from acetic acid (1 Gm. in 18 ml.) in fine needles which give off acetic acid around 135° with effervescence, then melt at 252.2–254.4° (X). A mixture of X with phenolphthalein (m. p. 261°) melts at 225–235°. These crystals from acetic acid dried first at 25°, then to 150° in vacuum, lose 17.4 ± 0.7% in weight, and the liquid coming off is acetic acid (Calcd. for  $C_{34}H_{22}O_7 \times 2 CH_3COOH$ : 18.1%).

*Anal.* of crystals obtained from acetic acid and dried at 25°.—Calcd. for  $C_{34}H_{22}O_7 \times 2 CH_3COOH$ : C, 68.88; H, 4.53. Found: C, 69.17 ± 0.87; H, 4.67 ± 0.14.

*Anal.* crystals, after drying at 150° *in vacuo*.—Calcd. for  $C_{34}H_{22}O_7$ : C, 75.27; H, 4.06; mol. wt. 542; three H, 0.55. Found: C, 75.55 ± 0.32; H, 4.46 ± 0.12; mol. wt. 583 ± 63; active H, 0.46.

These analytical figures also check with the empirical formulas  $C_{33}H_{21}O_7$  and  $C_{36}H_{26}O_7$ .

Compound X also can be recrystallized from acetone, forming rectangular crystals containing solvate acetone. It dissolves in 0.1 *N* sodium hydroxide with the same pink color as phenolphthalein in the same concentration.

When a solution of 0.1 Gm. of X in 0.5 ml. 5 *N* sodium hydroxide is heated in a sealed glass tube for twenty-four hours to 100°, most of X is recovered unchanged.

When 0.4 Gm. of X was added to 5 Gm. molten KOH at 230–235° and the melt stirred for five minutes, then phenol, benzoic acid, and *p*-hydroxybenzoic acid were obtained.

The red solution made up from 1.0 Gm. of X in 6.6 ml. 0.2 *N* sodium hydroxide was kept for four months in an atmosphere of oxygen at room temperature. The brownish solution was then found to contain *o*-(*p*-hydroxybenzoyl)-benzoic acid.

To a solution of 1.0 Gm. of X in 14 ml. sodium hydroxide, 2.0 ml. 30% hydrogen peroxide was added. The temperature went up slowly to a maximum of 47°, the color changed to a bluish-red, and a precipitate formed. The solution was saturated with  $CO_2$ . The water-soluble phenol consisted of 31 mg. hydroquinone. The water-insoluble brown phenol (0.33 Gm.) was acetylated and the acetyl-derivative recrystallized several times from ethanol; it melted at 149–151° (XI b) and the melting point was not depressed when mixed with the triacetyl-derivative of 3-(*p*-hydroxyphenyl)-3-(*m,p*-dihydroxyphenyl)-phthalide of m. p. 154.5–155.6°. Kin (6) gives an m. p. of 148° for this triacetyl-derivative. XI b on hydrolysis in 1 *N* sodium hydroxide yields a bluish solution, of the same shade as that of an alkaline phenol-catechol-phthalein.

Some phthalic acid was isolated also.

X reacts with acetic anhydride, but the acetyl-derivative could not be made to crystallize; neither

<sup>1</sup> All melting points are corrected. Molecular weights were determined by the Signer method, as described by Clark, E. P., *Ind. Eng. Chem., Anal. Ed.*, 13, 820 (1941).

could the gummy methyl-ether be obtained crystalline

**Dicarboxylic Acid XII**—A mixture of 10 Gm of X, 20 ml 80% acetic acid, and 10 Gm zinc (30 mesh) was refluxed. When all the zinc had dissolved, an additional 10 Gm zinc was added. The solution was poured into water and the gummy precipitate became hard. It was crystallized from 20% ethanol. This dicarboxylic acid melts at 258.9–259.8° (decompos).

*Anal*—Calcd for  $C_{17}H_{12}O_7$ : C, 74.72, H, 4.79, mol wt 516. Found: C, 74.15, H, 5.10, mol wt 510, neut equiv 280.

## Synthesis of 2-Benzyl-9-phenylxanthene

***o*-(*p*-Benzylphenoxy)benzoic Acid**.—The bluish solution of 15.6 Gm (0.1 mole) of *o*-chlorobenzoic acid (m p 133.5–140.0°), 18.4 Gm (0.1 mole) of 4-hydroxydiphenylmethane (m p 79–80°), 0.2 Gm CuCl in 100 ml 2 *N* of sodium methylate was heated on an oil bath. First, the methanol distilled off. The temperature was raised gradually to 200° (oil bath) and kept for one hour at that temperature. The crude acid, after one crystallization from 41% ethanol (1 Gm in 52 ml) weighed 22.4–24.4 Gm (yield 73–80%) and melted at 138–139°.

By subliming this acid at 135° and 8  $\mu$  pressure, it is obtained as white crystals, melting at 141.8–142.2°. Its solution in concentrated sulfuric acid is colorless but becomes bluish fluorescent on heating.

*Anal*—Calcd for  $C_{27}H_{16}O_3$ : C, 78.94, H, 5.26, mol wt 304. Found: C, 79.23, H, 5.41, mol wt 291, neut equiv 301.

The *p*-toluidide, purified by crystallizations from ethanol, melted at 119.2–121.3°.

*Anal*—Calcd for  $C_{27}H_{17}N_2O$ : C, 82.41, H, 5.89, N, 3.36. Found: C, 82.71, H, 5.76, N, 3.38.

The 2-naphthylamide of this acid, crystallized from ethanol, melted at 100.1–101.3°.

*Anal*—Calcd for  $C_{28}H_{17}NO_2$ : C, 83.89, H, 5.39, N, 3.26. Found: C, 83.81, H, 5.14, N, 3.42.

**2-Benzyl-9-xanthenone**—A solution of 30.0 Gm of *o*-(*p*-benzylphenoxy)benzoic acid, 80 ml of acetyl chloride, and 0.5 ml of concentrated sulfuric acid (7) was heated to a final temperature of 95°, distilling off HCl and excess acetyl chloride. On adding water to the cooled solution, the xanthenone crystallized out. It was dissolved in 800 ml ethanol at reflux temperature and then half of the solvent was distilled off. The yield was 26.0 Gm (92%), m p 132–134°. The same compound is formed when heating the *o*-aryloxybenzoic acid with a mixture of acetic anhydride and a small amount of concentrated sulfuric acid.

By sublimation at 130° and 8  $\mu$  pressure, and subsequent crystallization of the white sublimate from ethanol (1 Gm in 25 ml), the pure 2-benzyl-9-xanthenone is obtained as colorless needles, melting at 135.4–136.1°. It is soluble in benzene, but only slightly in ether. Its solution in concentrated sulfuric acid is yellow, with intense bluish fluorescence under ultraviolet light.

*Anal*—Calcd for  $C_{20}H_{14}O_2$ : C, 83.89, H, 4.92. Found: C, 84.33, H, 4.79.

**2-Benzyl-9-hydroxy-9-phenylxanthene (I)**—Grignard reagent (100% excess) made from 5 Gm magnesium, 15.7 Gm bromobenzene, and ml ether was added to a solution of 14.3 Gm benzyl-9-xanthenone in 120 ml benzene at 0°. The mixture was refluxed for one hour. After cooling and the addition of water, the solvent was steamed out. The remaining brownish-yellow solidified (18.8 Gm, m p 100–112°).

For purification, 1 Gm is dissolved in a mixture of 8 ml acetic acid and 2 ml 3 *N* hydrochloric acid and water added to the yellow filtrate. The droplets will solidify on warming (m p 112–120°). The hydrolyl may also be crystallized by adding 3 ml petroleum ether to a solution of 1 Gm in 2 ml benzene.

The pure compound (1) melts at 118–121°. Its solution in concentrated sulfuric acid is of strong yellow color with green fluorescence. It dissolves in concentrated hydrochloric acid as well as in 85% phosphoric acid with reddish orange color but without fluorescence.

*Anal*—Calcd for  $C_{26}H_{20}O$ : C, 85.71, H, 5.56. Found: C, 85.36, H, 5.64.

**2-Benzyl-9-ethoxy-9-phenylxanthene**.—When a solution of 6 Gm of I in 100 ml of ethanol is refluxed a short time, needles form on cooling. The melt at 80.7–81.7°. Their solution in concentrated sulfuric acid is yellow with green fluorescence.

*Anal*—Calcd for  $C_{28}H_{22}O$ : C, 85.66, H, 6.11. Found: C, 85.11, H, 6.30.

**2-Benzyl-9-phenylxanthene**.—A mixture of 1 Gm of hydrolyl I, 30 Gm sodium formate, and 1 ml 99% formic acid (8) was refluxed for one hour. The crystalline mass, obtained after the addition of water, was dissolved in 600 ml ethanol. After evaporation of 450 ml ethanol from the filtrate, crystals formed. These were then sublimed at 120–125° and 6  $\mu$  pressure. The white crystalline sublimate (7.07 Gm, yield 74%) melted at 122–125°.

Pure 2-benzyl-9-phenylxanthene, obtained as colorless crystals by further crystallization from ethanol (1 Gm in 55 ml) melted at 126.2–126.9°. It is insoluble in concentrated sulfuric acid at 25°.

*Anal*—Calcd for  $C_{26}H_{20}O$ : C, 89.62, H, 5.77. Found: C, 89.36, H, 5.67.

## Infrared Spectroscopy

The infrared spectra were evaluated by Dr F. Katlafsky of Monsanto Chemical Co, St Louis 24, Mo.

**Compound V**.—An overall similarity exists between phthalides and compound V, phenolphthalein having the closest, and fluoran the second best resemblance to V. *Ortho* and *para* substitution is found on the phenyl ring. The dilactone structure is substantiated, one carbonyl coinciding with the one of phenolphthalein and the other with the fluoran carbonyl. In the 3  $\mu$  section of compound V, there is a single free hydroxyl.

**Compound VI**.—A study of the infrared spectrum of compound of m p 125° shows that it is either an aryl ether or an unsaturated ether of the structure  $—C=C—O—C—$  such as occurs in xanthene. The strong bands at 13.2 and 14.4 indicate that *ortho*-disubstituted and -monosubstituted aromatic nuclei

are present. The absence of an absorption band at 3.5 of equal or greater intensity to the aromatic C—H band at 3.3 indicates that no CH<sub>2</sub> or CH<sub>3</sub> groups are present. The spectrum of VI resembles, in many respects, that of 9-phenylxanthene or 9-benzylxanthene.

**Compound X.**—The infrared spectrum shows a weak band at 11  $\mu$  which is absent in the spectrum of phenolphthalein and of isophenolphthalein. Since we are dealing with aromatic nuclei, this band could be interpreted as the vibration of one isolated ring hydrogen atom situated between substituents. The band for the vibration of such an isolated ring hydrogen atom in 1,2,4 and 1,3,5 trisubstituted and 1,2,3,5 and 1,2,4,5 tetrasubstituted aromatic

nuclei is expected to be weak. The spectrum also shows that the *ortho* disubstituted aromatic band at 13.2 increases in intensity with respect to its counterpart in the spectrum of phenolphthalein.

REFERENCES

(1) Hubacher, M. H., and Doernberg, S., *THIS JOURNAL*, 37, 261(1948).  
(2) Meyer, R., and Saul, E., *Ber.*, 2S, 3588(1892); Meyer, R., *Ber.*, 38, 450(1905).  
(3) Hubacher, M. H., *J. Am. Chem. Soc.*, 6S, 2097(1943).  
(4) Orndorff, W. R., and Murray, R. R., *J. Am. Chem. Soc.*, 39, 683(1917).  
(5) Clar, E., *Ber.*, 72, 1645(1939).  
(6) Kin, L. C., *Ann. chim.*, 13, 354(1940).  
(7) Gottesmann, E., *Ber.*, 66, 1168(1933).  
(8) Guyot, A., and Kovache, A., *Compt. rend.*, 1SS, 839 (1912).

Dosage Schedule and Pharmacokinetics  
in Chemotherapy\*

By EKKEHARD KRÜGER-THIEMER

The relationships between the chemotherapeutical dosage schedule (supporting dose  $D$ , ratio of initial to supporting dose  $D^+/D$ , dosing interval  $\tau$ ) and the pharmacokinetical constants  $k_1$ ,  $k_2$ , and  $V_d$ , the adsorption constants  $\alpha$  and  $\beta$ , and the minimum inhibition concentration  $\mu$  of the bacteriostatic drug, are formulated.

IN THE CHEMOTHERAPY with sulfanilamides and other bacteriostatic drugs, a therapeutically effective minimum concentration  $c_{min}$  of the drug in the blood plasma should be reached as soon as possible and maintained for the duration of the therapy by applying a dosage schedule consisting of an initial dose  $D^+$  and several lower supporting doses  $D$ , repeated with dosing intervals  $\tau$ . In this paper, the functional relationships between the dosage schedule and the plasma concentration  $c$ , influenced by the pharmacokinetics, the adsorption by the plasma proteins, and the bacteriostatic activity of the drug, are described. In the following equations there will be used the terms (units in parentheses):  $t$ , time after first drug administration ( $h$ );  $\tau$ , dosing interval ( $h$ );  $D/G$ , relative supporting dose (mg./Kg.);  $G$ , body weight of patient (Kg.);  $D^+/D$ , dose ratio

(mg./mg.);  $k_1$ , rate constant of invasion ( $h^{-1}$ );  $k_2$ , rate constant of elimination ( $h^{-1}$ );  $t_{50}$ , time of half elimination ( $h$ ) =  $\ln 2/k_2$ ;  $V_d$ , relative volume of distribution (ml./Gm.);  $\alpha$ , drug concentration in plasma ultrafiltrate at half saturation of adsorption ( $\mu$ mol./L);  $\beta$ , maximal specific adsorption by plasma proteins ( $\mu$ mol./Gm.);  $c$ , plasma concentration of the drug ( $\mu$ mol./L);  $c_0$ , extrapolated (fictive) initial concentration in the plasma ( $\mu$ mol./L);  $c_n$ , last measured value of concentration in the plasma ( $\mu$ mol./L);  $c'$ , concentration of freely dissolved drug in plasma water ( $\mu$ mol./L);  $M$ , molecular weight of drug (Gm./mol.);  $w$ , ratio of volume of plasma water to volume of whole plasma;  $p$ , concentration of proteins in blood plasma (Gm./L);  $\mu$ , minimum inhibition concentration against the infecting bacteria in a liquid medium free from antagonists ( $\mu$ mol./L);  $\sigma$ , proportionality constant in Eq. 1.

The following five assumptions are made: (a) According to Davis (1), the bacteriostatic activity of the drug in the blood depends on the concentration  $c'$  of the freely dissolved (ultrafiltrable) drug in the plasma water: this concentration  $c'_{min}$  necessary for effective therapy should be proportional to the minimum inhibition concentration  $\mu$

$c'_{min} = \sigma \cdot \mu$  (Eq. 1)

\* Received August 26, 1959, from the Tuberkuloseforschungsinstitut bei Bad Oldesloe, Holstein, Germany.  
of Advisory Council of the Tuberculosis Research, October 31, 1959.  
The author is indebted to Prof. Paul Burger and Werner Diller, Hamburg, and to Prof. Uecker, Berlin, and Prof. Hans G. O. Meyer, Berlin, for critical discussions.

where  $\sigma$  depends on the bacteriostatic, degenerative, or bactericidal character of the drug action and on the content of antagonists in body fluids. A rational method for the determination of  $\sigma$  does not exist;  $\sigma$  may be approximately obtained by comparison of clinical experience with bacteriological results.

(b) According to Witzgall (2) after reaching the equilibrium of distribution there will be equal concentrations  $c'$  of freely dissolved drug in the plasma water and in the water of all parts of the body, into which the drug may permeate.

$$c' = c'_1 = c'_2 = c'_3 = \dots \quad (\text{Eq. 2})$$

Therefore the conditions of drug action are equal in these parts of the body.

(c) The adsorption of the drug by plasma proteins is described by the adsorption isotherm of Langmuir (3), i. e., by the following relationship between the concentration  $c$  in the plasma and the concentration  $c'$  in the plasma ultrafiltrate of the bacteriostatic form of the drug

$$c = c' \cdot \left( w + \frac{\beta \cdot p}{\alpha + c'} \right) \quad (\text{Eq. 3})$$

(d) The time-plasma concentration relationship is described with sufficient accuracy according to Dost (4) by the equation

$$c = \frac{c_0 \cdot k_1}{k_1 - k_2} \cdot (e^{-k_2 t} - e^{-k_1 t}) \quad (\text{Eq. 4})$$

(e) According to Dost (4) and Boxer, *et al.* (5), the fictive initial concentration  $c_0$  is a function of  $D$ . The ratio of these two terms is called volume of distribution and, after division by the body weight  $G$ , relative volume of distribution  $V_d$

$$V_d = \frac{D}{c_0} \cdot \frac{1000}{G \cdot M} \quad (\text{Eq. 5})$$

In the usually applied range of doses of several sulfanilamides  $V_d$  is found to be a constant (6).

Dost (4) has given equations for the accumulation of a drug applied in several equal doses  $D$  at constant intervals  $\tau$ . Earlier approaches to this problem were made by Widmark and Tandberg (7), by Boxer, *et al.* (5), and by Druekrey and Küpfmüller (8). From Dost's equation for the lower limit of accumulation

$$c_{\min} = \frac{c_0 \cdot k_1}{k_1 - k_2} \cdot \left( \frac{1}{1 - e^{-k_2 \tau}} - \frac{1}{1 - e^{-k_1 \tau}} \right) \quad (\text{Eq. 6})$$

and from the above mentioned five assumptions the following equations for the dose ratio with extravasal application

$$\frac{D^+}{D} = \frac{1}{(1 - e^{-k_1 \tau}) \cdot (1 - e^{-k_2 \tau})} \quad (\text{Eq. 7})$$

and for the relative supporting dose  $D/G$

$$\frac{D}{G} = \frac{M \cdot \sigma \cdot \mu}{1000} \cdot \left( w + \frac{\beta \cdot p}{\alpha + \sigma \cdot \mu} \right) \cdot V_d \cdot \left( 1 - \frac{k_2}{k_1} \right) \cdot \frac{(1 - e^{-k_1 \tau}) \cdot (1 - e^{-k_2 \tau})}{e^{-k_1 \tau} - e^{-k_2 \tau}} \quad (\text{Eq. 8})$$

can be derived. Equation 7 is consistent within the range of doses having constant values of  $V_d$  (see Eq. 5). For intravenous application with  $k_1 \rightarrow \infty$ , the equations of the dosage schedule are

$$\frac{D^+}{D} = \frac{1}{1 - e^{-k_2 \tau}} \quad (\text{Eq. 9})$$

and

$$\frac{D}{G} = \frac{M \cdot \sigma \cdot \mu}{1000} \cdot \left( w + \frac{\beta \cdot p}{\alpha + \sigma \cdot \mu} \right) \cdot V_d \cdot (e^{k_2 \tau} - 1) \quad (\text{Eq. 10})$$

The equations for continuous intravenous infusion result from Eqs. 7 and 8 by  $k_1 \rightarrow \infty$  and  $\tau \rightarrow 0$  with  $D/t$  being the infusion rate

$$\frac{D^+}{D/t} = \frac{1}{k_2} \quad (\text{Eq. 11})$$

$$\frac{D}{G \cdot t} = \frac{M \cdot \sigma \cdot \mu}{1000} \cdot \left( w + \frac{\beta \cdot p}{\alpha + \sigma \cdot \mu} \right) \cdot V_d \cdot k_2 \quad (\text{Eq. 12})$$

The values of  $k_1$ ,  $k_2$ ,  $V_d$ ,  $\alpha$  and  $\beta$ , which are necessary for the calculation of  $D^+/D$  and  $D/G$ , may be obtained from any complete time-plasma concentration curve determined after a single dose  $D$  of the drug within the supposed therapeutically effective dose range, and from two (or better, more) estimates of the plasma ultrafiltrate concentration  $c'$  in relation to the plasma concentration  $c$  over a concentration range of 1 to 10.  $k_2$  is equal to the negative slope of the descending straight part of the time-log<sub>e</sub>  $c$  curve [Dost (4), Boxer, *et al.* (5), Swintosky, *et al.* (9)]. For the calculation of  $V_d$  from Eq. 5 and of  $k_1$  from Eq. 4, the value of  $c_0$  corresponding to the applied relative dose  $D/G$  must be determined. For this purpose, a relationship, recently found by Dost (10), may be used which connects the area  $F$  between the time-plasma concentration curve and the abscissa axis with  $c_0$  and  $k_2$

$$F = \int_0^\infty c \cdot dt = \frac{c_0}{k_2} \quad (\text{Eq. 13})$$

This follows from Eq. 4 by integration. If, at the end of the experiment ( $t = t_n$ ), the ordinate  $c_n$  is not negligible, the integration of the curve from 0 to  $t_n$  does not yield the whole integral of Eq. 13. As the deficient part of the area equals  $c_n/k_2$  in good approximation,  $c_0$  may be calculated from the equation.

$$c_0 = k_2 \cdot \int_0^{t_n} c \cdot dt + c_n \quad (\text{Eq. 14})$$

the integral in which may be obtained planimetrically or by calculation using the formula of sums of trapezes.  $k_1$  may be gained from Eq. 4 by series evolution for  $e^{-(k_1-k_2)t}$ , whereby three approximations may be calculated, explicitly, the first and second of which are

$$k_1' = \frac{c}{t_0 \cdot e^{-k_2 t}} \tag{Eq. 15}$$
$$k_1'' = \frac{1}{t} \cdot \left( 1 + \frac{k_2 \cdot t}{2} \pm \sqrt{\left( 1 + \frac{k_2 \cdot t}{2} \right)^2 - 2 \cdot k_1' \cdot t} \right) \tag{Eq. 16}$$

It is of interest, that the influence of  $k_1$ , which is quite variable at peroral application, on Eqs. 7 and 8 is small. Therefore, in most cases, the first approximation (Eq. 15) calculated with that one of the first measured values, which gives the greatest value of  $c/t$ , is sufficient. The two constants of adsorption  $\alpha$  and  $\beta$  may be calculated from many pairs of values  $c_1, c_1'$ , using transformed Eq. 3 with Gauss' method of least squares of errors; in the graph of the straight line,  $\alpha$  is given by the negative intercept with the ordinate axis and  $\beta$  is given by the slope of the line (abscissa =  $p/[(c/c') - w]$  and ordinate =  $c'$ ).

Before  $D^+/D$  and  $D/G$  can be calculated, a suitable value of  $\tau$  has to be selected. For clinical convenience, only proper fractions of twenty-four hours should be taken. From an investigation of many sulfanilamide derivatives (6), it may be mentioned that  $D^+/D$  in the clinic is often taken as 2.0. In most of these cases,  $\tau$  nearly equals the time of half elimination  $t_{50}$ . This relation which was found empirically follows also from Eq. 7 for  $\tau = t_{50}$ , and a great value of  $k_1$ . Therefore, it may be stated that the suitable value of the dosing interval  $\tau$  should be near to the value of the time of half elimination  $t_{50}$  of the drug; in this case, the dose ratio  $D^+/D$  nearly equals 2.0. Only the relative supporting dose  $D/G$  remains to be calculated numerically from Eq. 8. Nomograms for these calculations will be published elsewhere (11). For the calculation of  $D/G$ , the value of  $\sigma$  is necessary. As already mentioned, an independent method for the determination of  $\sigma$  is not available. By comparison of clinically used and calculated relative supporting doses  $D/G$  a value of  $\sigma = 2$  was found for *Escherichia coli* and several sulfanilamide derivatives (11).

The upper limit of the relative supporting dose  $D/G$  is given as the toxicity of the drug. Current definitions of the chronic toxicity do not take

into consideration the pharmacokinetics of the drug, so that it seems to be necessary to define a new criterion of chronic toxicity: Dosis sustinentis tolerata 95 per cent ( $DST_{95}$ , mg./Kg.) is defined as the supporting dose of a dosage schedule (with  $D^+/D = 2.0$  and  $\tau = t_{50}$ ), which is tolerated without toxic symptoms by 95 per cent of the treated individuals; the method for estimation of  $DST_{95}$  is analogous to that described by Wagner (12) for the dosis curativa 95 per cent ( $DC_{95}$ ).

From the presented pharmacokinetical theory of the dosage schedule it may be concluded that a full description of the chemotherapeutical properties of a drug requires at least eight values: the three pharmacokinetical constants  $k_1$ ,  $k_2$ , and  $V_d$ ; the two adsorption constants,  $\alpha$  and  $\beta$ ; the minimum inhibition concentration  $\mu$ ; the factor  $\sigma$ , and the dosis sustinentis tolerata 95%  $DST_{95}$ . These constants may achieve clinical significance only in association with their standard deviations obtained from many (10 or more) patients or experimental animals. The possibility of calculating  $D^+/D$  and  $D/G$  by Eqs. 7 to 12 is very restricted by the difficulties of the estimation of  $\sigma$  and  $DST_{95}$ . In the author's opinion, the main use of these equations is to clarify the mathematical structure of the interrelationships between the properties and the therapeutical effect of a drug, whereby the number of the biological constants necessary for the description of these interrelationships is determined. These biological constants will be useful in comparing various drugs with an analogous mode of action. An application of this pharmacokinetical theory of dosage schedule on new sulfanilamide derivatives (6) and a detailed description of the implications of this theory (11) will be published elsewhere.

REFERENCES

(1) Davis, B. D., *Science*, 95, 78(1942)

(2) Witzgall, H., *Arztliche Wochschr.*, 8, 643(1953)

(3) Langmuir, I., *J. Am. Chem. Soc.*, 39, 1848(1917).

(4) Dost, F. H., "Der Blutspiegel, Kinetik der Konzentrationsabläufe in der Kreislaufblutigkeit," Georg Thieme-Verlag, Leipzig, Germany, 1953, pp. 41, 122, 132, 255

(5) Boxer, G. E., Jelinek, V. C., Tompsett, R., DuBois, R., and Edison, A. O., *J. Pharmacol. Exptl. Therap.*, 92, 226 (1948)

(6) Bünger, P., Führ, J., Krüger-Thiemer, E., and Diller, W., *Arzneimittel-Forsch.* 10, in press

(7) Widmark, E., and Tandberg, J., *Biochem. Z.*, 147, 358(1924)

(8) Druckrey, H., and Küpfmüller, K., "Dosis und Wirkung, Beiträge zur theoretischen Pharmakologie," Aulendorf, Württemberg, Germany, 1949

(9) ... .., *THIS JOURNAL*, 4S, 395(1956);

.. .., M. J., Foltz, E. L., and Free,

Wochschr., 36, 655(1958).

E., *Jahresbericht Borstel*, vol. 5, in

press, and *Arztl. Wochschr.*, in press

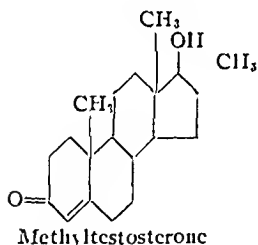
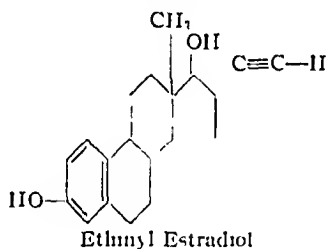
(12) Wagner, W.-H., *Arzneimittel-Forsch.*, 3, 66(1953).

# Spectrophotometric Assay for Combinations of Ethinyl Estradiol and Methyltestosterone\*

By SHELDON KLEIN, ARTHUR E. JAMES, and MURRAY M. TUCKERMAN

The ultraviolet absorption curves of ethinyl estradiol and of methyltestosterone were determined in acidic and alkaline methanol. The methyltestosterone absorption curve is independent of pH in the range studied; however the absorption of ethinyl estradiol is affected by changes in the pH of the solvent. Ethinyl estradiol has a stable absorption maximum at 281  $m\mu$  in acidic methanol and a stable maximum at 298  $m\mu$  in alkaline methanol solutions greater than 0.2 *N* in potassium hydroxide. At ratios of ethinyl estradiol to methyltestosterone in the order of 6:1 to 1:4, simultaneous determination of the two is satisfactory. In commercial products in which the ratio is of the order 1:200 to 1:500, methyltestosterone can be determined directly, whereas acceptable results for ethinyl estradiol appear to require separation.

METHYLTESTOSTERONE has been included in the U S P since 1947, ethinyl estradiol since 1955. The objective of the work described herewith was to develop a simultaneous assay for these two hormones. Their structural formulas may be represented as shown



For the identification and the quantitative analysis of ethinyl estradiol, fluorescence, colorimetric, ultraviolet, and infrared measurements have been suggested (1-5). For methyltestosterone, colorimetric, ultraviolet, polarographic, and angular rotation measurements have been used (2, 6-16).

\* Received August 21, 1959, from Temple University, School of Pharmacy, Philadelphia 40, Pa.

Based, in part, upon a thesis presented by Sheldon Klein to the Faculty, Temple University, School of Pharmacy, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

Presented to the Scientific Section, A Ph A, Cincinnati meeting, August 1959.

The authors wish to thank the Schering Corp., Bloomfield, N. J., Ciba Pharmaceutical Products, Inc., Summit, N. J., and Warren Teed Products Co., Columbus, Ohio, for their generous supply of products and information for this work. Also, E. R. Squibb and Sons, Division of Olin Mathieson Chemical Corp., New Brunswick, N. J., Chicago Pharmacal Co., Chicago, Ill., and Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N. Y., for their generous supply of products used for this work.

## EXPERIMENTAL

**Absorptivity of Methyltestosterone.**—Measurements of U S P Reference Standard methyltestosterone absorption in methanol 1 *N* and 0.01 *N* in hydrochloric acid, and in methanol 0.1 *N*, 0.3 *N*, and 1 *N* in potassium hydroxide, showed that the absorption curve was unaffected by the pH of the solvent. The absorption maximum is at 241  $m\mu$ . At this wavelength, absorption follows Beer's law over the range 2 to 20 mg/ml;  $E(1\%, 1\text{ cm}) = 508$ . This value is not in accord with the U. S. P. figure of 520-540 for an ethanolic solution. At 298  $m\mu$  Beer's law is followed in the concentration range 1 to 5 mg/ml;  $E(1\%, 1\text{ cm}) = 330$ . The absorption curves are shown in Figs 1 and 2. The absorptivity values,  $E(1\%, 1\text{ cm})$  are shown in Tables I and II. In this and subsequent work each value is the average of two or more determinations.

TABLE I—ABSORPTIVITY OF METHYLTESTOSTERONE IN 0.01 *N* HCl-CH<sub>3</sub>OH AT 241  $m\mu$

Concentration Methyltestosterone, mg/ml	Absorbance at 241 $m\mu$	Absorptivity, $E(1\%, 1\text{ cm})$
2.00	0.102	510
5.00	0.252	504
10.00	0.509	509
20.00	1.020	510
		Average 508

TABLE II—ABSORPTIVITY OF METHYLTESTOSTERONE IN 0.3 *N* KOH-CH<sub>3</sub>OH AT 298  $m\mu$

Concentration Methyltestosterone, mg/ml	Absorbance at 298 $m\mu$	Absorptivity, $E(1\%, 1\text{ cm})$
1.00	0.315	315
2.00	0.660	330
5.00	1.653	331
		Average 325

**Absorptivity of Ethinyl Estradiol.**—In acid methanol the absorptivity maximum of U S P ethinyl estradiol is at 281  $m\mu$ ,  $E(1\%, 1\text{ cm}) = 686$ . Beer's law is followed over the concentration range 10 to 50

107 mg/ml. In alkaline methanol, the absorption maximum shifts gradually from 281  $m\mu$  to 298  $m\mu$  as the concentration of potassium hydroxide is increased, until a stable absorption maximum is reached at a concentration about 0.2 *N* potassium hydroxide. At potassium hydroxide concentrations between 0.2 *N* and 1 *N* the absorption maximum is at 298  $m\mu$ ;  $E(1\%, 1\text{ cm.}) = 90$ . Absorption curves are shown in Figs. 1 and 2. The absorptivity values are shown in Tables III and IV. The data for the absorptivity of ethinyl estradiol is given at 241  $m\mu$  this being the maximum absorption of methyltestosterone.

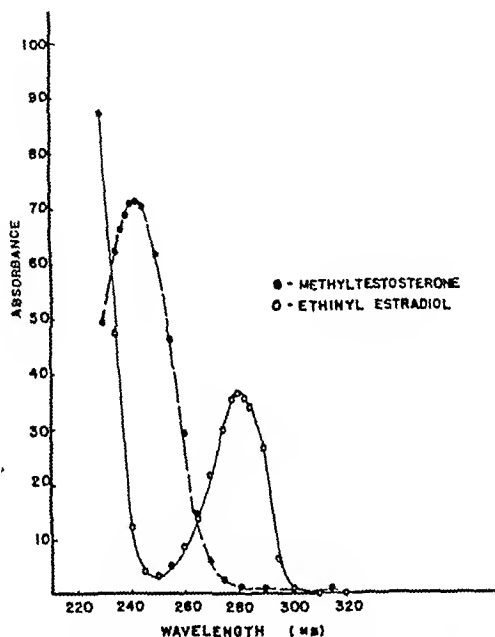


Fig. 1—Absorption curves in 1*N* HCl-CH<sub>3</sub>OH

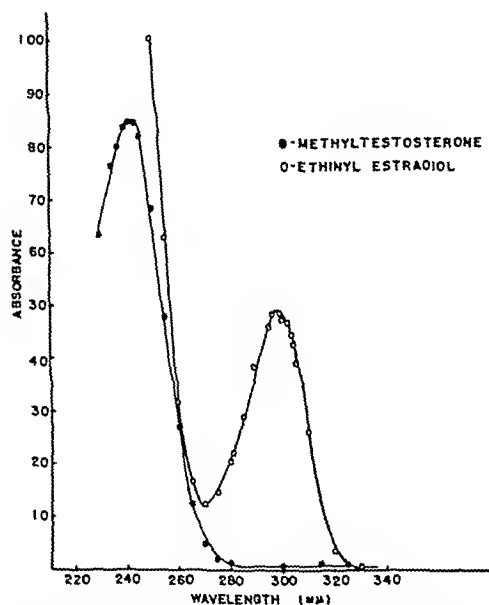


Fig. 2—Absorption curves in 1*N* KOH-CH<sub>3</sub>OH

TABLE III—ABSORPTIVITY OF ETHINYL ESTRADIOL IN 0.01 *N* HCl-CH<sub>3</sub>OH AT 241  $m\mu$

Concentration Ethinyl Estradiol, mg/ml	Absorbance at 241 $m\mu$	Absorptivity $E(1\%, 1\text{ cm})$
18.8	0.034	18.0
37.5	0.068	18.0
75.0	0.132	17.6
		Average 18.0

TABLE IV—ABSORPTIVITY OF ETHINYL ESTRADIOL IN VARIOUS ALKALINE METHANOL SOLUTIONS AT 298  $m\mu$

Solvent	$E(1\%, 1\text{ cm})$
0.01 <i>N</i> KOH-CH <sub>3</sub> OH	56.0
0.05 <i>N</i> KOH-CH <sub>3</sub> OH	86.0
0.1 <i>N</i> KOH-CH <sub>3</sub> OH	88.0
0.2 <i>N</i> KOH-CH <sub>3</sub> OH	90.2
0.3 <i>N</i> KOH-CH <sub>3</sub> OH	90.4
0.5 <i>N</i> KOH-CH <sub>3</sub> OH	90.6

**Standard Series.**—A series of mixtures in chloroform was prepared in which the ratio of the concentrations of ethinyl estradiol to methyltestosterone was varied from 6:1 to 1:1. Aliquots were evaporated and taken up in methanol 0.01 *N* in hydrochloric acid and in methanol 0.3 *N* in potassium hydroxide. Another series was prepared by separately dissolving the hormones in methanol. Aliquots of each solution were taken to prepare a series of mixtures containing a ratio of concentrations of ethinyl estradiol to methyltestosterone from 1:1 to 1:5. One aliquot of each mixture was diluted with methanol 0.01 *N* in hydrochloric acid, a second, with methanol 0.3 *N* in potassium hydroxide.

**Simultaneous Spectrophotometric Determination of Standards.**—The absorbance of the acidic solutions was measured at 241  $m\mu$ , that of the basic solutions at 298  $m\mu$ , see Table V. The concentrations of each hormone were calculated by the method of simultaneous equations (17). These equations reduce to the following forms:

$$\text{concentration of ethinyl estradiol (mg/ml)} = \frac{(51 A_{298} - 0.33 A_{241})/458}{\text{and concentration of methyltestosterone (mg/ml)} = \frac{A_{241} - 1.8 \times \text{concentration of ethinyl estradiol (mg/ml)}/51}$$

$A$  refers to the measured absorbance of the solution, the subscript denotes the wavelength at which the measurement was made. All measurements were made in a cell with a path of 1 cm.

**Assay of Commercial Products for Methyltestosterone.**—Current pharmaceutical products containing mixtures of ethinyl estradiol and methyltestosterone in the order of 1:200 to 1:500 were assayed for methyltestosterone without separation of the hormones. Aliquots of powdered tablets equivalent to 120 mg of testosterone were extracted with three successive portions of ether (U. S. P.). The combined ether extracts were washed with three successive 10-ml portions of 10% NaHCO<sub>3</sub>, followed by two successive 10-ml portions of 0.1 *N* HCl. The ether extract was made up to a volume of 100 ml. with this solvent. Two-milliliter portions of the



TABLE V—SIMULTANEOUS ASSAY OF KNOWN MIXTURES OF ETHINYL ESTRADIOL AND METHYLTESTOSTERONE IN 0.01 N HCl-CH<sub>3</sub>OH AND 0.3 N KOH-CH<sub>3</sub>OH

Solution	Ethinyl Estradiol			Methyltestosterone		
	Theoretical, mg/ml	Experimental, mg/ml	Error, %	Theoretical, mg/ml	Experimental, mg/ml	Error, %
A	30.0	29.9	-0.3	4.88	4.57	-6.3
B	45.0	45.2	+0.4	9.76	9.57	-2.0
C	15.0	15.2	+1.3	4.96	4.92	-0.8
D	15.0	15.0	0.0	9.92	9.75	-1.7
E	15.0	15.3	+2.0	12.2	11.8	-3.3
F	5.11	5.04	-1.4	5.00	5.00	0.0
G	5.11	5.30	+3.7	10.0	9.98	-0.2
H	5.11	5.15	+0.8	20.0	19.8	-1.0
I	5.11	5.76	+12.7	25.0	24.6	-1.6

ether solution were evaporated to dryness. The residue was taken up in methanol 0.1 N in HCl and made up to a volume of 250 ml. The absorbance of this solution was determined at 211 m $\mu$ . The methyltestosterone in mg/ml =  $A_{211}/50.8$ .

Suitable volumes of liquid preparations containing the two hormones in the above-mentioned ratios were extracted with ether. The combined ether extracts were washed with distilled water and made up to a volume of 100 ml with ether. Two-milliliter aliquots were evaporated to dryness and the residue taken up in 250 ml of methanol 0.1 N in HCl. The absorbance of the resulting solutions was measured at 241 m $\mu$  and the methyltestosterone content calculated by the same formula used for tablets. Typical results are shown in Table VI.

TABLE VI—METHYLTESTOSTERONE IN COMMERCIAL PRODUCTS

Product	Labelled Content	Experimental	Deviation, %
Tablets A	10 mg/tablet	9.6	4.0
Tablets B	5 mg/tablet	4.5	10.0
Liquid X	2.5 mg/ml	2.3	8.0

## DISCUSSION

The simultaneous spectrophotometric assay is based on the absorption of solutions in acidic and alkaline methanol. Though the absorption curve of methyltestosterone appears stable throughout the pH range investigated, the absorption curve for ethinyl estradiol depends upon the degree of ionization of its phenolic group. This group appears

to be unionized in acidic methanol and to be completely ionized in alkaline methanol greater than 0.2 N in potassium hydroxide.

Methyltestosterone is calculated from the absorbance in acidic methanol in order to minimize interference from ethinyl estradiol. Ethinyl estradiol is calculated from the absorbance in alkaline methanol in order to minimize interference from methyltestosterone and to increase the sensitivity of the assay, since ethinyl estradiol has its absorption maximum at a longer wavelength and has a higher absorbance in alkaline methanol than in acidic methanol.

## REFERENCES

- (1) Rosecott, R. J., *Nature*, 162, 577(1948).
- (2) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1955.
- (3) Jelinek, P. H., *Nature*, 171, 750(1953).
- (4) Carol, J. J. *Assoc. Offic. Agr. Chemists*, 40, 837 (1957).
- (5) Miller, L. C., private communication.
- (6) McCullagh, D. R., Schneider, I., and Emery, F., *Ludocrinology*, 27, 71(1940).
- (7) Diding, E., *Svensk Farm. Tidsskr.*, 56, 3(1952), *Chem. Abstr.*, 46, 6325i(1952).
- (8) McKinley, W. P., and Devlin, W. F., *Can. J. Biochem. and Physiol.*, 35, 699(1957).
- (9) Johnston, C. D., *Science*, 106, 91(1947).
- (10) Sartori, G., and Bianchi, E., *Gazz. chim. ital.*, 74, 8(1944), *Chem. Abstr.*, 42, 1266h(1947).
- (11) Pasez, M., *Bull. soc. chim. France*, 1947, 911, *Chem. Abstr.*, 42, 2545h(1948).
- (12) Carol, J. J., *J. Assoc. Offic. Agr. Chemists*, 34, 572(1951).
- (13) Sjostrom, E., and Nykjaer, L., *THIS JOURNAL*, 46, 321(1957).
- (14) Schering Corp., Bloomfield, N. J., private communication.
- (15) The Warren-Teed Products Co., Columbus, Ohio, private communication.
- (16) Ciba Pharmaceutical Products Inc., Summit, N. J., private communication.
- (17) Kress, K. E., *Anal. Chem.*, 23, 312(1951).

# Some Ketonic Mannich Bases\*

By ELIZABETH D. TAYLOR and W. LEWIS NOBLES

A group of ketonic Mannich bases, represented by Types I and II, were synthesized for pharmacological evaluation. Some of these agents demonstrated *in vitro* anti-tubercular and amebicidal action.

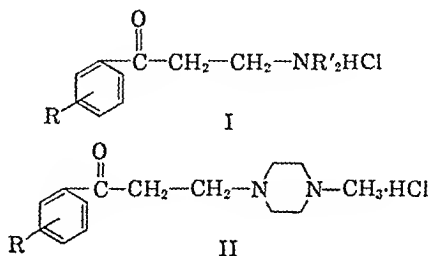
INTEREST HAS BEEN EXPRESSED in the pharmacological action of various ketonic Mannich bases (1-8). The extensive literature dealing with this reaction has been reviewed recently by Reichert (9).

Mannich and Lammering reported that  $\beta$ -piperidinopropiophenone hydrochloride possessed local anesthetic activity (1); it was observed by others (2) that the similar amino analog from 2-acetylpyrrole demonstrated this same type of activity. It has been indicated by Levvy and Nisbet (3) that both the piperidino and dimethylamino Mannich bases of 2-acetylthiophene possessed local anesthetic activity; subsequently, Denton and his associates (4) described the antispasmodic activity of compounds of this type and other closely related structures.

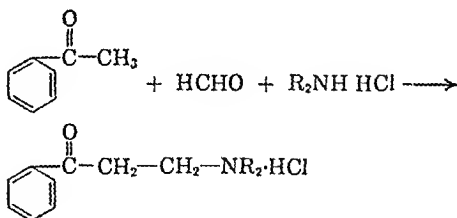
Burckhalter and Johnson (5) reported the antibacterial activity of certain Mannich bases derived from  $\alpha,\beta$ -unsaturated ketones. We have previously reported analogs of some of these more active compounds (6). Mercier and his associates indicated that the diethylamino Mannich base from acetophenone was adrenolytic, hypotensive and ganglioplegic (7). More recently, Issekutz and co-workers (8) reported that certain substituted propiophenones of this type inhibited nicotine tremors and convulsions in rabbits, and raised the lethal dose of nicotine in mice. The 3-diethylamino compound was the most potent compound reported in this study; in addition to this type of activity, this compound inhibited water diuresis in rats when administered at a dosage level of 5 mg/100 Gm. body weight. Several of the agents reported in this study were weak spasmolytic agents when tested on isolated rabbit and guinea pig intestinal strips.

In view of this multiplicity of pharmacological effects elicited by these simple ketonic Mannich bases, we wished to prepare a number of previously unreported Mannich bases of Type I, especially

those containing halogen substituents in view of the well known effect of halogen substituents in increasing activity in many compounds demonstrating these general types of pharmacological activity. In addition, the recent interest in the pharmacological activity of piperazine and its derivatives prompted us to prepare compounds of Type II, in which the amine moiety is the disubstituted piperazine.



The preparations of compounds of Types I and II, which are listed in Tables I and II, was accomplished by means of the Mannich reaction, which involved the interaction of formaldehyde or paraformaldehyde with the appropriate ketone and amine hydrochloride. This reaction may be depicted as follows:



## EXPERIMENTAL

**Experimental Results.**—*p*-Fluoroacetophenone and *p*-iodoacetophenone were prepared according to the method of Lutz (10). *p*-Chlorobenzalacetone was synthesized according to the method previously outlined (11). The N-methylpiperazine was kindly supplied through the courtesy of Dr. Harold Zaugg of Abbott Laboratories. All the other ketones and amines used in this study were commercially available.

The Mannich reaction was carried out as previously described (6) utilizing the two procedures therein alluded to depending upon the physical state in which the amine or its hydrochloride was usually available. In general, the reactions proceeded smoothly to yield the expected product. It may be noted, however, that excellent yields are seldom obtained in the Mannich reaction due to the complexity of the products obtained which may be occasioned by by-product formation.

\* Received August 21, 1959, from the University of Mississippi, School of Pharmacy, University.

This paper was based on a portion of a thesis submitted by Mrs. Elizabeth D. Taylor in partial fulfillment of the requirements for the Master of Science degree in the Graduate School of the University of Mississippi, August 1955.

Presented to the Scientific Section A. Ph. A., Cincinnati meeting, August 1959.

TABLE V.—SIMULTANEOUS ASSAY OF KNOWN MIXTURES OF ETHINYL ESTRADIOL AND METHYLTESTOSTERONE IN 0.01 N HCl-CH<sub>3</sub>OH AND 0.3 N KOH-CH<sub>3</sub>OH

Solution	Ethinyl Estradiol			Methyltestosterone		
	Theoretical, meg/ml	Experimental meg/ml	Error, %	Theoretical, meg/ml	Experimental, meg/ml	Error, %
A	30.0	29.9	-0.3	4.88	4.57	-6.3
B	45.0	45.2	+0.4	9.76	9.57	-2.0
C	15.0	15.2	+1.3	4.96	4.92	-0.8
D	15.0	15.0	0.0	9.92	9.75	-1.7
E	15.0	15.3	+2.0	12.2	11.8	-3.3
F	5.11	5.04	-1.4	5.00	5.00	0.0
G	5.11	5.30	+3.7	10.0	9.98	-0.2
H	5.11	5.15	+0.8	20.0	19.8	-1.0
I	5.11	5.76	+12.7	25.0	24.6	-1.6

ether solution were evaporated to dryness. The residue was taken up in methanol 0.1 N in HCl and made up to a volume of 250 ml. The absorbance of this solution was determined at 241  $\mu$ . The methyltestosterone in mg./ml. =  $A_{241}/50.8$ .

Suitable volumes of liquid preparations containing the two hormones in the above-mentioned ratios were extracted with ether. The combined ether extracts were washed with distilled water and made up to a volume of 100 ml with ether. Two-milliliter aliquots were evaporated to dryness and the residue taken up in 250 ml of methanol 0.1 N in HCl. The absorbance of the resulting solutions was measured at 241  $\mu$  and the methyltestosterone content calculated by the same formula used for tablets. Typical results are shown in Table VI.

TABLE VI — METHYLTESTOSTERONE IN COMMERCIAL PRODUCTS

Product	Labelled Content	Experimental	Deviation, %
Tablets A	10 mg./tablet	9.6	4.0
Tablets B	5 mg./tablet	4.5	10.0
Liquid X	2.5 mg./ml	2.3	8.0

## DISCUSSION

The simultaneous spectrophotometric assay is based on the absorption of solutions in acidic and alkaline methanol. Though the absorption curve of methyltestosterone appears stable throughout the pH range investigated, the absorption curve for ethinyl estradiol depends upon the degree of ionization of its phenolic group. This group appears

to be unionized in acidic methanol and to be completely ionized in alkaline methanol greater than 0.2 N in potassium hydroxide.

Methyltestosterone is calculated from the absorbance in acidic methanol in order to minimize interference from ethinyl estradiol. Ethinyl estradiol is calculated from the absorbance in alkaline methanol in order to minimize interference from methyltestosterone and to increase the sensitivity of the assay, since ethinyl estradiol has its absorption maximum at a longer wavelength and has a higher absorbance in alkaline methanol than in acidic methanol.

## REFERENCES

- (1) Boscott, R. J., *Nature*, **162**, 577(1918).
- (2) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1935.
- (3) Jellinek, P. H., *Nature*, **171**, 750(1953).
- (4) Carol, J., *J. Assoc. Offic. Agr. Chemists*, **40**, 837(1957).
- (5) Miller, L. C., private communication.
- (6) McCullagh, D. R., Schneider, I., and Emery, F., *Endocrinology*, **27**, 71(1940).
- (7) Didling, E., *Siensk. Farm. Tidskr.*, **56**, 3(1952); *Chem. Abstr.*, **46**, 6325i(1952).
- (8) McKinley, W. P., and Devlin, W. F., *Can. J. Biochem. and Physiol.*, **35**, 699(1957).
- (9) Johnston, C. D., *Science*, **106**, 91(1947).
- (10) Sartori, G., and Bianchi, E., *Gazz. chim. ital.*, **74**, 8(1914); *Chem. Abstr.*, **42**, 1266h(1947).
- (11) Pasce, M., *Bull. soc. chim. France*, 1947, 911; *Chem. Abstr.*, **42**, 2515h(1948).
- (12) Carol, J., *J. Assoc. Offic. Agr. Chemists*, **34**, 572(1951).
- (13) Sjostrom, E., and Nykanen, L., *This Journal*, **46**, 321(1957).
- (14) Schering Corp., Bloomfield, N. J., private communication.
- (15) The Warren-Teed Products Co., Columbus, Ohio, private communication.
- (16) Ciba Pharmaceutical Products Inc., Summit, N. J., private communication.
- (17) Kress, K. E., *Anal. Chem.*, **23**, 312(1951).

# Some Ketonic Mannich Bases\*

By ELIZABETH D. TAYLOR and W. LEWIS NOBLES

A group of ketonic Mannich bases, represented by Types I and II, were synthesized for pharmacological evaluation. Some of these agents demonstrated *in vitro* anti-tubercular and amebicidal action.

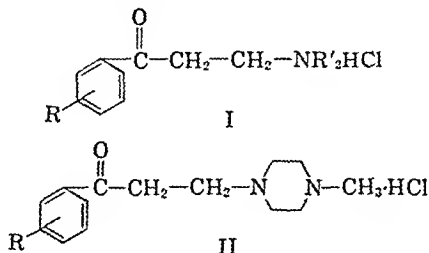
INTEREST HAS BEEN EXPRESSED in the pharmacological action of various ketonic Mannich bases (1-8). The extensive literature dealing with this reaction has been reviewed recently by Reichert (9).

Mannich and Lammering reported that  $\beta$ -piperidinopropiophenone hydrochloride possessed local anesthetic activity (1); it was observed by others (2) that the similar amino analog from 2-acetylpyrrole demonstrated this same type of activity. It has been indicated by Levvy and Nisbet (3) that both the piperidino and dimethylamino Mannich bases of 2-acetylthiophene possessed local anesthetic activity; subsequently, Denton and his associates (4) described the antispasmodic activity of compounds of this type and other closely related structures.

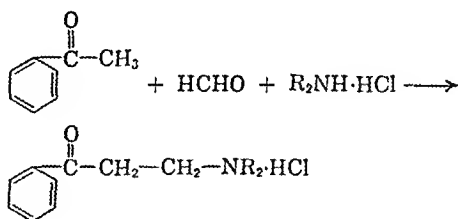
Burckhalter and Johnson (5) reported the antibacterial activity of certain Mannich bases derived from  $\alpha,\beta$ -unsaturated ketones. We have previously reported analogs of some of these more active compounds (6). Mercier and his associates indicated that the diethylamino Mannich base from acetophenone was adrenolytic, hypotensive and ganglioplegic (7). More recently, Issekutz and co-workers (8) reported that certain substituted propiophenones of this type inhibited nicotine tremors and convulsions in rabbits, and raised the lethal dose of nicotine in mice. The 3-diethylamino compound was the most potent compound reported in this study; in addition to this type of activity, this compound inhibited water diuresis in rats when administered at a dosage level of 5 mg./100 Gm. body weight. Several of the agents reported in this study were weak spasmolytic agents when tested on isolated rabbit and guinea pig intestinal strips.

In view of this multiplicity of pharmacological effects elicited by these simple ketonic Mannich bases, we wished to prepare a number of previously unreported Mannich bases of Type I, especially

those containing halogen substituents in view of the well known effect of halogen substituents increasing activity in many compounds demonstrating these general types of pharmacological activity. In addition, the recent interest in the pharmacological activity of piperazine and its derivatives prompted us to prepare compounds of Type II, in which the amine moiety is the disubstituted piperazine.



The preparations of compounds of Types I and II, which are listed in Tables I and II, was accomplished by means of the Mannich reaction, which involved the interaction of formaldehyde or paraformaldehyde with the appropriate ketone and amine hydrochloride. This reaction may be depicted as follows:



## EXPERIMENTAL

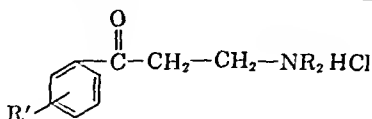
**Experimental Results.**—*p*-Fluoroacetophenone and *p*-iodoacetophenone were prepared according to the method of Lutz (10). *p*-Chlorobenzalacetone was synthesized according to the method previously outlined (11). The N-methylpiperazine was kindly supplied through the courtesy of Dr. Harold Zaugg of Abbott Laboratories. All the other ketones and amines used in this study were commercially available.

The Mannich reaction was carried out as previously described (6) utilizing the two procedures therein alluded to depending upon the physical state in which the amine or its hydrochloride was usually available. In general, the reactions proceeded smoothly to yield the expected product. It may be noted, however, that excellent yields are seldom obtained in the Mannich reaction due to the complexity of the products obtained which may be occasioned by by-product formation.

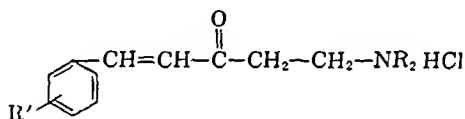
\* Received August 21, 1959, from the University of Mississippi, School of Pharmacy, University.

This paper was based on a portion of a thesis submitted by Mrs. Elizabeth D. Taylor in partial fulfillment of the requirements for the Master's degree at the University of Mississippi. Presented to the Scientific meeting, August 1959.

TABLE I —MANNICH BASES



No	R'	NR <sub>2</sub>	Yield, %	M <sub>o</sub> P, °C	Formula	Analyses %		Hydrogen %	
						Calcd	Found	Calcd	Found
1	<i>p</i> -Fluoro	Dimethylamino	48	156-158	C <sub>11</sub> H <sub>11</sub> FNO HCl	57.00	57.01	6.52	6.39
2	<i>p</i> -Fluoro	<i>n</i> -Dibutylamino	42	189-190	C <sub>17</sub> H <sub>23</sub> FNO HCl	61.64	64.83	8.61	8.49
3	<i>p</i> -Fluoro	Piperidino	68	191	C <sub>14</sub> H <sub>19</sub> FNO HCl	61.87	61.63	7.05	7.45
4	<i>p</i> -Fluoro	Morpholino	65	215-217	C <sub>13</sub> H <sub>17</sub> FNO <sub>2</sub> HCl	57.04	56.80	6.26	6.16
5	<i>p</i> -Fluoro	(2-Methyl)-piperidino	22	205-206	C <sub>15</sub> H <sub>21</sub> FNO HCl	63.26	62.81	7.43	7.23
6	<i>p</i> -Fluoro	Pyrrolidino	38	145	C <sub>11</sub> H <sub>16</sub> FNO HCl	60.58	60.30	6.65	7.08
7	<i>p</i> -Iodo	Dimethylamino	42	208-210	C <sub>11</sub> H <sub>11</sub> INO HCl	38.90	38.96	4.45	4.61
8	<i>p</i> -Iodo	Piperidino	52	214-216	C <sub>14</sub> H <sub>19</sub> INO HCl	44.28	44.20	5.04	5.16
9	<i>p</i> -Iodo	Morpholino	47	209-210	C <sub>13</sub> H <sub>17</sub> INO <sub>2</sub> HCl	40.91	40.87	4.49	4.61
10	<i>p</i> -Iodo	(2-Methyl)-piperidino	26	209-210	C <sub>15</sub> H <sub>21</sub> INO HCl	45.87	45.63	5.39	5.22
11	<i>p</i> -Iodo	Pyrrolidino	31	199-200	C <sub>11</sub> H <sub>16</sub> INO HCl	42.70	42.76	4.68	4.79
12	<i>o</i> -Hydroxy <sup>a</sup>	Dimethylamino	33	175-176	C <sub>11</sub> H <sub>15</sub> NO HCl				
13	<i>o</i> -Hydroxy	Piperidino	65	180	C <sub>14</sub> H <sub>19</sub> NO <sub>2</sub> HCl	62.33	61.71	7.49	7.38
14	<i>o</i> -Hydroxy	Morpholino	59	193-195	C <sub>13</sub> H <sub>17</sub> NO <sub>3</sub> HCl	57.45	57.31	6.68	6.49
15	<i>o</i> -Hydroxy	(2-Methyl)-piperidino	57	210-212	C <sub>15</sub> H <sub>21</sub> NO <sub>2</sub> HCl	63.48	62.95	7.81	7.68
16	<i>o</i> -Hydroxy	Pyrrolidino	28	161-162	C <sub>13</sub> H <sub>17</sub> NO <sub>2</sub> HCl	61.05	60.59	7.09	7.28



17	<i>p</i> -Chloro	Piperidino	60	198-200	C <sub>14</sub> H <sub>19</sub> ClNO HCl	61.15	61.35	6.73	6.53
18	<i>p</i> -Methoxy	(2-Methyl)-piperidino	75	212-213	C <sub>15</sub> H <sub>21</sub> NO <sub>2</sub> HCl	66.75	66.28	8.12	8.34

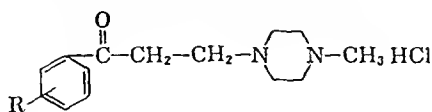
<sup>a</sup> All Mannich bases in this table were recrystallized from an ethanol acetone solution

<sup>b</sup> Melting points are uncorrected

<sup>c</sup> Carbon and hydrogen analyses are by Weiler and Strauss, Oxford, England

<sup>d</sup> Padfield, E. M., and Tomlinson, M. L. *J. Chem. Soc.* 1950, 2272 report a m. p. of 176° for a compound which they indicate to be the dimethylamino Mannich base, although in their description they refer to the use of methylamine. The nature of their investigation would dictate that it was in fact the dimethylamino compound they were utilizing in connection with the work reported in the reference cited.

TABLE II —MANNICH BASES



No	R	Yield %	M <sub>o</sub> P, °C	Formula	Analyses %		Hydrogen %	
					Calcd	Found	Calcd	Found
1	<i>p</i> -Fluoro	78	181	C <sub>14</sub> H <sub>19</sub> FNO <sub>2</sub> 2HCl H <sub>2</sub> O	49.27	49.86	6.79	6.54
2	<i>p</i> -Chloro	80	179-180	C <sub>14</sub> H <sub>19</sub> ClNO <sub>2</sub> 2HCl H <sub>2</sub> O	47.00	46.69	6.48	6.61
3	<i>p</i> -Bromo	68	185-187	C <sub>14</sub> H <sub>19</sub> BrNO <sub>2</sub> 2HCl 3H <sub>2</sub> O	38.39	38.82	6.21	6.18
4	<i>p</i> -Iodo	77	176-177	C <sub>14</sub> H <sub>19</sub> INO <sub>2</sub> 2HCl 4H <sub>2</sub> O	33.44	33.48	5.81	6.05
5	None <sup>c</sup>	58	180-181	C <sub>14</sub> H <sub>20</sub> N <sub>2</sub> O 2HCl H <sub>2</sub> O	52.01	52.10	7.48	7.16
6	<i>p</i> -Nitro	71	192-194	C <sub>14</sub> H <sub>19</sub> N <sub>3</sub> O <sub>3</sub> 2HCl H <sub>2</sub> O	45.66	45.93	6.29	6.41
7	<i>p</i> -Methoxy	81	190-192	C <sub>15</sub> H <sub>21</sub> N <sub>2</sub> O <sub>2</sub> 2HCl 2H <sub>2</sub> O	48.29	48.43	7.56	7.60
8	<i>p</i> -Ethoxy	70	187-188	C <sub>16</sub> H <sub>23</sub> N <sub>2</sub> O <sub>2</sub> 2HCl H <sub>2</sub> O	52.32	52.51	7.68	7.40
9	<i>m</i> -Nitro	38	170	C <sub>14</sub> H <sub>19</sub> N <sub>3</sub> O <sub>3</sub> 2HCl H <sub>2</sub> O	45.66	45.38	6.29	6.16

<sup>a</sup> Melting points are uncorrected

<sup>b</sup> Carbon and hydrogen analyses are by Weiler and Strauss, Oxford, England

<sup>c</sup> Cymerman, Craig, J. A.

which melts at 197° this *et al.* *J. Am. Chem. Soc.* 71

melting point of 187.1-188.5° It is of interest that all of the present compounds were obtained as hydrates when the usual Mannich procedure was used with recrystallization from ethanol acetone

8, 378(1955) refer to an anhydrous form of this compound after the completion of the present work. Denton, J. J. with 0.75 mole of water of hydration for which they report

TABLE III.—TUBERCULOSIS SCREENING, RESULTS *in Vitro*, SERUM BROTH DILUTION

Compound	Organism	Strain	Incnb., Days	Meg./ml. Causing Complete Inhibition	Partial Inhibition	Standard Control (INH) meg./ml. Causing Complete Inhibition
1. No. 1	<i>M. tuberculosis</i>	...	Inactive	..	..	...
2. No. 9	<i>M. tuberculosis</i>	...	Inactive	..	..	...
3. No. 10	<i>M. tuberculosis</i>	H37RV	7	20	..	0.024
4. No. 10	<i>M. tuberculosis</i>	INH-Res.	7	20	..	...
5. No. 10	<i>M. tuberculosis</i>	STM-Res.	7	20	..	...
6. No. 13	<i>M. tuberculosis</i>	H37RV	7	20	..	0.024
7. No. 13	<i>M. tuberculosis</i>	INH-Res.	7	20	10	...
8. No. 13	<i>M. tuberculosis</i>	STM-Res.	7	20	10	...

TABLE IV.—AMEBICIDAL ACTIVITY

Compound	Dilution	Drug Effect
No. 1	1:2500	Cidal
No. 1	1:5000	Cidal
No. 9	1:5000	Cidal
No. 13	1:2500	Cidal
No. 13	1:5000	Stasis

**Pharmacological Results.**—The results of the pharmacological evaluation of these compounds have been made available through the courtesy of Dr. Loren Long of Parke, Davis and Co. All of the compounds thus far tested are from Table I and the numbers used in this section will refer to the numbers cited in that table only. The *in vitro* antitubercular and amebicidal activities of some of these compounds are listed in Tables III and IV.

Despite the *in vitro* activity of compounds No. 10 and 13 in the tuberculosis screening test, these compounds were regarded as being inactive in subsequent *in vivo* tests. Also, compound No. 9 was not regarded as being promising in subsequent *in vivo* amebicidal tests which were conducted. Compound No. 11 did not demonstrate any *in vivo* activity against either *Strep. pyogenes* or *Staph. aureus*.

Moreover, compound No. 1 evidenced no respiratory stimulation in doses ranging from 2–32 mg./Kg. when administered intravenously to cats. No cerebral stimulation was noted with this compound in doses up to 100 mg./Kg. The same negative results were elicited by compounds No. 8 and 13. A mild degree of respiratory stimulation was elicited, however, by compound No. 9 when it was

administered to cats at a dosage level of 4–8 mg./Kg., but no cerebral stimulation was evidenced with this agent at doses up to 100 mg./Kg.

Compound No. 1 produced stasis in *in vitro* tests against *Trichomonas vaginalis* at a concentration of 50 meg./ml. but was inactive at a level of 12.5 meg./ml.

## SUMMARY

Twenty-seven Mannich bases have been prepared, using various ketones with dialkyl and heterocyclic amines. While several of these compounds did demonstrate *in vitro* activity against various organisms as reported in this paper, subsequent *in vivo* tests of these substances were not regarded as promising in any instance.

## REFERENCES

- (1) Mannich, C., and Lammering, D., *Ber.*, 55, 3510 (1922).
- (2) Blicke, F. F., and Blake, E. S., *J. Am. Chem. Soc.*, 52, 235 (1930).
- (3) Levvy, G. A., and Nisbet, H. B., *J. Chem. Soc.*, 1938, 1053.
- (4) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *J. Am. Chem. Soc.*, 71, 2048, 2050, 2053, 2054 (1949); 72, 3279, 3792 (1950).
- (5) Burckhalter, J. H., and Johnson, S. H., *ibid.*, 73, 4835 (1951).
- (6) Nobles, W. L., *et al.*, *THIS JOURNAL*, 43, 641 (1954); 43, 644 (1954); 44, 273 (1955); 44, 717 (1955); 47, 77 (1958).
- (7) Mercier, P., *et al.*, *J. Physiol. Paris*, 45, 186 (1953).
- (8) Issekutz, B., *et al.*, *Acta Physiol. Acad. Sci. Hung.*, 6, 95 (1954).
- (9) Reichert, B., "Die Mannich-Reaction," Deutscher Apotheker Verlag, Stuttgart, Germany, 1959.
- (10) Lutz, R. E., *et al.*, *J. Org. Chem.*, 12, 617 (1947).
- (11) Dodgen, D., and Nobles, W. L., *THIS JOURNAL*, 46, 437 (1957).

TABLE I—MANNICH BASES

$\text{R}'-\text{C}_6\text{H}_4-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-\text{NR}_2\cdot\text{HCl}$									
No. <sup>a</sup>	R'	NR <sub>2</sub>	Yield, %	M. P. <sup>b</sup> °C	Formula	Analyses, %			
						Carbon		Hydrogen	
						Calcd	Found	Calcd	Found
1	<i>p</i> -Fluoro	Dimethylamino	48	156-158	C <sub>11</sub> H <sub>14</sub> FNO·HCl	57.00	57.01	6.52	6
2	<i>p</i> -Fluoro	<i>n</i> -Dibutylamino	42	189-190	C <sub>17</sub> H <sub>27</sub> FNO·HCl	64.64	64.83	8.61	8
3	<i>p</i> -Fluoro	Piperidino	68	191	C <sub>11</sub> H <sub>16</sub> FNO·HCl	61.87	61.63	7.05	7
4	<i>p</i> -Fluoro	Morpholino	65	215-217	C <sub>11</sub> H <sub>16</sub> FNO <sub>2</sub> ·HCl	57.04	56.80	6.26	6.1
5	<i>p</i> -Fluoro	(2-Methyl)-piperidino	22	205-206	C <sub>15</sub> H <sub>20</sub> FNO·HCl	63.26	62.81	7.43	7.2
6	<i>p</i> -Fluoro	Pyrrolidino	38	145	C <sub>11</sub> H <sub>16</sub> FNO·HCl	60.58	60.30	6.65	7.0
7	<i>p</i> -Iodo	Dimethylamino	42	208-210	C <sub>11</sub> H <sub>14</sub> I·NO·HCl	38.90	38.96	4.45	4.6
8	<i>p</i> -Iodo	Piperidino	52	214-216	C <sub>11</sub> H <sub>16</sub> I·NO·HCl	44.28	44.20	5.04	5.1
9	<i>p</i> -Iodo	Morpholino	47	209-210	C <sub>11</sub> H <sub>16</sub> I·NO <sub>2</sub> ·HCl	40.91	40.87	4.49	4.6
10	<i>p</i> -Iodo	(2-Methyl)-piperidino	26	209-210	C <sub>15</sub> H <sub>19</sub> I·NO·HCl	45.87	45.63	5.39	5.2
11	<i>p</i> -Iodo	Pyrrolidino	34	199-200	C <sub>11</sub> H <sub>16</sub> I·NO·HCl	42.70	42.76	4.68	4.79
12	<i>o</i> -Hydroxy <sup>d</sup>	Dimethylamino	33	175-176	C <sub>11</sub> H <sub>13</sub> NO·HCl	..	..	..	..
13	<i>o</i> -Hydroxy	Piperidino	65	180	C <sub>11</sub> H <sub>16</sub> NO <sub>2</sub> ·HCl	62.33	61.71	7.49	7.38
14	<i>o</i> -Hydroxy	Morpholino	59	193-195	C <sub>11</sub> H <sub>17</sub> NO <sub>3</sub> ·HCl	57.45	57.31	6.68	6.49
15	<i>o</i> -Hydroxy	(2-Methyl)-piperidino	57	210-212	C <sub>15</sub> H <sub>21</sub> NO <sub>2</sub> ·HCl	63.48	62.95	7.81	7.68
16	<i>o</i> -Hydroxy	Pyrrolidino	28	161-162	C <sub>11</sub> H <sub>17</sub> NO <sub>2</sub> ·HCl	61.05	60.59	7.09	7.28
$\text{R}'-\text{C}_6\text{H}_4-\text{CH}=\text{CH}-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-\text{NR}_2\cdot\text{HCl}$									
17	<i>p</i> -Chloro	Piperidino	69	198-200	C <sub>11</sub> H <sub>12</sub> ClNO·HCl	61.15	61.35	6.73	6.58
18	<i>p</i> -Methoxy	(2-Methyl)-piperidino	75	212-213	C <sub>15</sub> H <sub>22</sub> NO <sub>2</sub> ·HCl	66.75	66.28	8.12	8.34

<sup>a</sup> All Mannich bases in this table were recrystallized from an ethanol-acetone solution.<sup>b</sup> Melting points are uncorrected.<sup>c</sup> Carbon and hydrogen analyses are by Weiler and Strauss, Oxford, England.<sup>d</sup> Padfield, E. M., and Tomlinson, M. L., *J. Chem. Soc.* 1950, 2272 report a m. p. of 176° for a compound which they indicate to be the dimethylamino Mannich base, although in their description they refer to the use of methylamine. The nature of their investigation would dictate that it was, in fact, the dimethylamino compound they were utilizing in connection with the work reported in the reference cited.

TABLE II—MANNICH BASES

No	R	Yield, %	M. P., °C	Formula	Analyses, %			
					Carbon		Hydrogen	
					Calcd	Found	Calcd	Found
1	<i>p</i> -Fluoro	78	181	C <sub>11</sub> H <sub>16</sub> FN <sub>2</sub> O·2HCl·H <sub>2</sub> O	49.27	49.86	6.79	6.54
2	<i>p</i> -Chloro	80	179-180	C <sub>11</sub> H <sub>15</sub> ClN <sub>2</sub> O·2HCl·H <sub>2</sub> O	47.00	46.69	6.48	6.61
3.	<i>p</i> -Bromo	68	185-187	C <sub>11</sub> H <sub>15</sub> BrN <sub>2</sub> O·2HCl·3H <sub>2</sub> O	38.39	38.82	6.21	6.18
4	<i>p</i> -Iodo	77	176-177	C <sub>11</sub> H <sub>15</sub> IN <sub>2</sub> O·2HCl·4H <sub>2</sub> O	33.44	33.48	5.81	6.05
5	None <sup>c</sup>	58	180-181	C <sub>14</sub> H <sub>20</sub> N <sub>2</sub> O·2HCl·H <sub>2</sub> O	52.01	52.10	7.48	7.16
6	<i>p</i> -Nitro	71	192-194	C <sub>11</sub> H <sub>13</sub> N <sub>3</sub> O <sub>5</sub> ·2HCl·H <sub>2</sub> O	45.66	45.93	6.29	6.41
7	<i>p</i> -Methoxy	81	190-192	C <sub>15</sub> H <sub>22</sub> N <sub>2</sub> O <sub>2</sub> ·2HCl·2H <sub>2</sub> O	48.29	48.43	7.56	7.60
8	<i>p</i> -Ethoxy	70	187-188	C <sub>16</sub> H <sub>24</sub> N <sub>2</sub> O <sub>2</sub> ·2HCl·H <sub>2</sub> O	52.32	52.51	7.68	7.40
9.	<i>m</i> -Nitro	38	170	C <sub>14</sub> H <sub>19</sub> N <sub>3</sub> O <sub>5</sub> ·2HCl·H <sub>2</sub> O	45.66	45.38	6.29	6.16

<sup>a</sup> Melting points are uncorrected.<sup>b</sup> Carbon and hydrogen analyses are by Weiler and Strauss, Oxford, England.<sup>c</sup> Cymerman-Craig, J., and Harrison, R. J., *Australian J. Chem.* 8, 378(1955), refer to an anhydrous form of this compound which melts at 197°; this reference was obtained from *Chem. Abstr.* after the completion of the present work. Denton, J. J., *J. Am. Chem. Soc.*, 71, 2048(1949), report a similar compound with 0.75 mole of water of hydration for which they report a melting point of 187.1-188.5°. It is of interest that all of the present compounds were obtained as hydrates when the usual Mannich procedure was used with recrystallization from ethanol-acetone.

TABLE III.—TUBERCULOSIS SCREENING, RESULTS *in Vitro*, SERUM BROTH DILUTION

Compound	Organism	Strain	Incub., Days	Meg. ml Causing Complete Inhibition	Partial Inhibition	Standard Control (INH) meg./ml Causing Complete Inhibition
1. No. 1	<i>M. tuberculosis</i>	...	Inactive			
2. No. 9	<i>M. tuberculosis</i>	...	Inactive			
3. No. 10	<i>M. tuberculosis</i>	H37RV	7	20		0.024
4. No. 10	<i>M. tuberculosis</i>	INH-Res.	7	20		
5. No. 10	<i>M. tuberculosis</i>	STM-Res.	7	20		
6. No. 13	<i>M. tuberculosis</i>	H37RV	7	20		0.024
7. No. 13	<i>M. tuberculosis</i>	INH-Res.	7	20	10	
8. No. 13	<i>M. tuberculosis</i>	STM-Res.	7	20	10	

TABLE IV.—AMEBICIDAL ACTIVITY

Compound	Dilution	Drug Effect
No. 1	1:2500	Cidal
No. 1	1:5000	Cidal
No. 9	1:5000	Cidal
No. 13	1:2500	Cidal
No. 13	1:5000	Stasis

**Pharmacological Results.**—The results of the pharmacological evaluation of these compounds have been made available through the courtesy of Dr. Loren Long of Parke, Davis and Co. All of the compounds thus far tested are from Table I and the numbers used in this section will refer to the numbers cited in that table only. The *in vitro* antitubercular and amebicidal activities of some of these compounds are listed in Tables III and IV.

Despite the *in vitro* activity of compounds No. 10 and 13 in the tuberculosis screening test, these compounds were regarded as being inactive in subsequent *in vivo* tests. Also, compound No. 9 was not regarded as being promising in subsequent *in vivo* amebicidal tests which were conducted. Compound No. 11 did not demonstrate any *in vivo* activity against either *Strep. pyogenes* or *Staph. aureus*.

Moreover, compound No. 1 evidenced no respiratory stimulation in doses ranging from 2–32 mg./Kg. when administered intravenously to cats. No cerebral stimulation was noted with this compound in doses up to 100 mg./Kg. The same negative results were elicited by compounds No. 8 and 13. A mild degree of respiratory stimulation was elicited, however, by compound No. 9 when it was

administered to cats at a dosage level of 4–8 mg./Kg., but no cerebral stimulation was evidenced with this agent at doses up to 100 mg./Kg.

Compound No. 1 produced stasis in *in vitro* tests against *Trichomonas vaginalis* at a concentration of 50 meg./ml. but was inactive at a level of 12.5 meg./ml.

## SUMMARY

Twenty-seven Mannich bases have been prepared, using various ketones with dialkyl and heterocyclic amines. While several of these compounds did demonstrate *in vitro* activity against various organisms as reported in this paper, subsequent *in vivo* tests of these substances were not regarded as promising in any instance.

## REFERENCES

- (1) Mannich, C., and Lammering, D., *Ber.*, 55, 3510 (1922).
- (2) Blicke, F. F., and Blake, E. S., *J. Am. Chem. Soc.*, 52, 235(1930).
- (3) Levvy, G. A., and Nisbet, H. B., *J. Chem. Soc.*, 1938, 1053.
- (4) Denton, J. J., Turner, R. J., Neier, W. B., Lawson, V. A., and Schedl, H. P., *J. Am. Chem. Soc.*, 71, 2048, 2050, 2053, 2054(1949); 72, 3279, 3792(1950).
- (5) Burekhalter, J. H., and Johnson, S. H., *ibid.*, 73, 4835(1951).
- (6) Nobles, W. L., *et al.*, *THIS JOURNAL*, 43, 641(1954); 43, 644(1954); 44, 717(1955); 47, 77(1958).
- (7) Mercier, F., *ibid.*, 5, 186(1953).
- (8) Issekutz, B., *Sci. Hung.*, 6, 95(1954).
- (9) Reichert, B., "Die Mannich-Reaktion," Deutscher Apotheker Verlag, Stuttgart, Germany, 1959.
- (10) Lutz, R. E., *et al.*, *J. Org. Chem.*, 12, 617(1947).
- (11) Dodgen, D., and Nobles, W. L., *THIS JOURNAL*, 46, 437(1957).



# Quantitative Determination of Ethanol in Pharmaceutical Products by Gas Chromatography\*

By HAROLD J. WESSELMAN

The presently used U. S. P. XV method for ethanol is compared with the gas chromatography method. Advantages and disadvantages of both methods are discussed. Typical results obtained by both procedures when applied to tinctures, fluidextracts, elixirs, and other products are compared. The results obtained from three gas chromatographs each having a different type thermal conductivity detector are presented. Three types of sample injectors are compared and five different stationary phases are examined.

SINCE MANY pharmaceutical preparations contain ethanol, it is desirable to use a method for the determination of ethanol which is both fast and accurate. The presently used distillation method described in the U. S. P. XV (1) is time consuming, at least one hour per assay, and often complicated by frothing and bumping during the distillation. Samples containing glycerin, iodine, volatile oils, solvents, or ammonia require special treatment prior to distillation. The determination of the specific gravity of the distillate offers another possibility for error.

The successful application of gas chromatography in previous work (2) suggested that it might be used for ethanol. Preliminary work showed that it offers an ideal solution to the above mentioned problems as samples can be assayed with little or no treatment in a short time with equal or better accuracy.

## EXPERIMENTAL

**Stationary Phase.**—In the search for a suitable stationary phase polyethylene glycol 400, diglycerol dimonyl phthalate, Ucon 75 H 90000, and triethyl phosphate were examined. Polyethylene glycol 400 was finally selected since it resolves ethanol, water, and acetone completely and the retention times are short, an important factor when many samples are to be assayed.

**Sample Injector.**—Initially, the peak height of ethanol in the sample being assayed was compared to the peak height of a standard ethanol solution. Good results were obtained, but the problem of repetitive injection of small samples (1 to 5  $\mu$ L) was very tedious.

The Hamilton microliter syringe No. 705N proved to be the most useful of the three injectors available since it is easy to use and to clean. The Agla microliter syringe was satisfactory but requires time to assemble, load, inject sample, disassemble, and clean. The final system used was that of Tenney and Harris (3). This system proved satisfactory although some liquids are difficult to load into the capillary pipets. Each of these injectors gave comparable results.

\* Received August 21, 1959 from the Analytical Control Research and Development Departments, Eli Lilly and Co., Indianapolis, Ind.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

TABLE I—RATIO OF PEAK HEIGHTS OF ETHANOL AND ACETONE FOR STANDARD SOLUTIONS OF ETHANOL AND ACETONE

Preparation	Ethanol, %	Acetone %	Ratio Ethanol/Acetone
Elixir Betalin <sup>a</sup> complex	13	10	1.04
Elixir Betalin <sup>a</sup> complex	14	10	1.10
Elixir Betalin <sup>a</sup> complex	15	10	1.15
Elixir Betalin <sup>a</sup> complex	16	10	1.21
Elixir Betalin <sup>a</sup> complex	17	10	1.27
F. E. Casarea sagrada aromatic	12	10	1.15
F. E. Casarea sagrada aromatic	14	10	1.23
F. E. Casarea sagrada aromatic	16	10	1.31
F. E. Casarea sagrada aromatic	18	10	1.39
F. E. Casarea sagrada aromatic	20	10	1.47
Elixir Amytal <sup>b</sup>	24	20	0.935
Elixir Amytal <sup>b</sup>	26	20	0.993
Elixir Amytal <sup>b</sup>	28	20	1.055
Elixir Amytal <sup>b</sup>	30	20	1.114
Elixir Amytal <sup>b</sup>	32	20	1.171
Fluid rose, soluble	30	30	0.7512
Fluid rose, soluble	32	30	0.7850
Fluid rose, soluble	34	30	0.8242
Fluid rose, soluble	36	30	0.8653
Fluid rose, soluble	38	30	0.9000
Tincture belladonna	44	30	1.026
Tincture belladonna	46	30	1.060
Tincture belladonna	48	30	1.104
Tincture belladonna	50	30	1.133
Tincture belladonna	52	30	1.171
Spirit camphor	70	10	4.125
Spirit camphor	72	10	4.177
Spirit camphor	74	10	4.250
Spirit camphor	76	10	4.275
Spirit camphor	78	10	4.324

<sup>a</sup> Betalin complex, vitamin B complex, Lilly.

<sup>b</sup> Amytal, amobarbital, Lilly.

**Calibration.**—Since the accuracy of the direct measurement of peak heights for quantitative determinations of ethanol depends mainly on the ability to inject accurate volumes of samples, this point was given serious consideration. The internal normalization technique cannot be applied because of the numerous volatiles and nonvolatiles found in the samples encountered. The internal standard technique introduced by Ray (4) and later used by Bradford, Harvey, and Chalkley (5) was then used since it is the most accurate of all methods,  $\pm 1\%$  being easily achieved. In this method a suitable

TABLE II.—COMPARISON OF ETHANOL DETERMINATIONS BY DISTILLATION AND GAS CHROMATOGRAPHY

Preparation	Theory, %	Distillation, %	Gas Chromatography, %			
			Analyst 1	Analyst 2	Average	Standard Deviation
Elixir Bctalin complex	17 00	17 27	16 90	17 33	17 27	0 97
			17 16	16 66		
			17 77	17 78		
			18 18	17 77		
F E Cascara sagrada aromatic	18 00	18 00	17 90	17 85	17 78	0 52
			17 50	17 50		
			33 55	33 33		
			34 22	33 60		
Elixir Amytal	34 00	32 88	34 10	34 20	33 83	0 91
			48 88	47 77		
			49 55	48 57		
			48 00	48 71		
Tincture belladonna U S P	67 00	67 14	66 66	68 57	67 55	2 45
			68 43	66 11		
			68 40	67 14		
			85 33	82 11		
Spirit camphor N F.	83 00	86 00	82 44	81 88	83 26	3 70
			83 15	84 64		

volatile substance is added to the sample in known proportion and the peak height of the ethanol is referred to the peak height of the internal standard. Calibration curves are obtained by measuring the ratio of the peak heights of ethanol and the internal standard and plotting this ratio against the percentage of ethanol. This provides straight line plots. Hausdorff (6) found that peak heights are linear with concentration when symmetrical and sharp peaks are obtained, and that peak heights are generally as accurate as peak areas.

Acetone was selected as an internal standard after examining methanol, isopropanol, butanol, and pentanol. Accurate measurement of the sample is no longer critical with this method. Brealey, Elvidge, and Proctor (7) reported that doubling or tripling the sample size does not appreciably alter the ratio of the peak heights. Since a calibration curve is run with each unknown, it is not necessary to maintain precise operating conditions in the instrument from time to time. The slope of the curve will change slightly from day to day, but it is only necessary to run two concentrations of the standard solutions to determine the curve.

Ratios of ethanol peak heights to acetone peak heights for the synthetic mixtures of several preparations are shown in Table I. The resulting calibration curves are shown in Fig 1.

### PROCEDURE

Standard solutions containing acetone and a suitable amount of ethanol are prepared for each sample to be assayed. The sample is diluted with the same amount of acetone that is contained in the standards. Standards and samples are then chromatographed under the following conditions: instrument, Podbielniak Chromacon No 9475-3V; column, 6 feet  $\times$   $1/4$  inch copper packed with 30% (w/w) polyethylene glycol 400 on 30-60 mesh Chromosorb and operated at 100°; sample inlet heater, temperature maintained at 300°; carrier gas, helium at an inlet pressure of 15 pounds/square inch and atmospheric pressure at outlet, flow rate 75 ml/min; pipet, Hamilton microliter syringe No 705N, sample size 1-2  $\mu$ L., detector, Gow-Mac

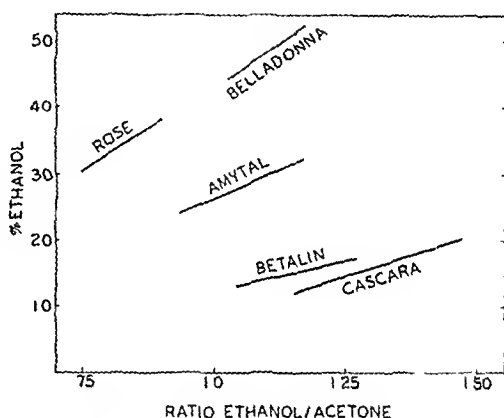


Fig 1—Calibration curves for ethanol determination

thermal conductivity cell, model 9285, detector current 68 milliamperes, output signal 32, recorder, Brown instruments division, Minneapolis-Honeywell Regulator Co with a range of  $-0.2-2.0$  mv and a chart speed of 30 inches/hour.

The peak heights of the acetone and ethanol are measured accurately and the ratios calculated and plotted against the concentration of ethanol. From the standard plot the concentration of ethanol in the diluted sample is determined. This value, when corrected for the dilution with acetone gives the amount of ethanol present in the original sample. Table II shows the results obtained by the distillation and gas chromatography methods.

In addition to the above mentioned instrument, a Burrell Kromo-Tog, model K-2, with a filament-type detector and a shop-built instrument with a Victory Engineering Corp thermistor-type detector, No M 182, were also used. The results from these instruments compared very favorably with those shown in Table II.

### SUMMARY

The gas chromatography method permits a sample containing ethanol to be assayed accu-

rately in less than one half hour as compared to at least one hour by the distillation method. No separations or special treatments are required in the former procedure, the sample needs only to be diluted with a known amount of acetone before being injected into the chromatograph. Results obtained by two analysts are very satisfactory.<sup>1</sup>

<sup>1</sup> The author wishes to thank Mr. G. W. Mills for assisting in the collection of some of these data.

## REFERENCES

- (1) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1955, p. 918.
- (2) Tepe, J. B., and Wesselman, H. J., *THIS JOURNAL*, 47, 457 (1958).
- (3) Tenney, H. M., and Harris, R. J., *Anal. Chem.*, 29, 317 (1957).
- (4) Ray, N. H., *J. Appl. Chem.*, 4, 21 (1954).
- (5) Bradford, B. W., Harvey, D., and Chalkley, D. E., *J. Inst. Petrol.*, 41, 80 (1955).
- (6) Hausdorff, H. H., "Vapour Phase Chromatography," Academic Press Inc., New York, N. Y., 1957, p. 377.
- (7) Brealey, L., Elvidge, D. A., and Proctor, K. A., *Analyst*, 84, 221 (1959).

# Metal Chelates and Diabetogenic Activity II\*

## Isoalloxazines

By WINTHROP E. LANGE, JAMES McMURTRY, and MERLE E. AMUNDSON†

In a further attempt to relate the chemical structure of various compounds to their diabetogenic activity, copper chelates of several isoalloxazines, and nitrogen-containing fragments (substituted anilines) of the isoalloxazine molecules, have been prepared. All of these compounds were found to be weak chelating agents. Only copper was found to be capable of forming stable 1:1 chelates. The chelates of dichloro-isoalloxazines as well as the chelates of the corresponding substituted aniline compounds were found to produce a hyperglycemic response in rabbits greater than that of alloxan or its metal chelates. The chelates of dimethyl-isoalloxazines and the corresponding substituted anilines as well as the unmetallized compounds were essentially inactive. These findings suggest that either chloro-substitution, or the presence of a sugar side chain is necessary for isoalloxazines to produce a hyperglycemic response as metal chelates.

KUHN AND WEYGAND (1) in 1934 were the first to experiment with isoalloxazine derivatives as antimetabolites. Since that time many isoalloxazines have been prepared and tested as: riboflavin antagonists (2), inhibitors of flavo-protein enzymes (3), and antifolinic compounds (4). Binet, Wellers, and Marquis (5) were the first to observe that large doses of various alloxazines produced a diabetogenic response in rats. This result is not surprising in view of the facts that the pyrimidine structure,

considered essential for diabetogenic activity (6), is present and of the large doses employed.

In the first paper in this series (7) we reported the preparation and blood sugar studies of various chelates of alloxan. At that time we also tested the chelates of two isoalloxazines, one of which produced a blood sugar response far greater than that of alloxan and the other showed only slight activity. In order to inquire further into the cause of the variations in response we obtained, copper chelates of various isoalloxazines and fragments of the isoalloxazine molecule have been prepared and tested in rabbits.

## DISCUSSION

**Methods of Synthesis.**—In order to determine the influence of substitution on the benzene portion of the isoalloxazine molecule, two derivatives of aniline were prepared. The preparation of the

\* Received August 21, 1959, from the Massachusetts College of Pharmacy, Boston, and the Division of Pharmacy, South Dakota State College, Brookings.

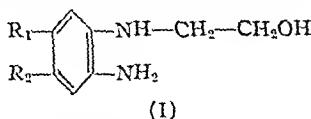
† Fellow of the American Foundation for Pharmaceutical Education, 1958-1959.

A portion of this work was abstracted from a thesis submitted by Merle E. Amundson as partial fulfillment of the requirements for the degree of Master of Science, 1959.

This study was supported in part by a research grant (A-1895) from the National Institutes of Health, U. S. Public Health Service.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

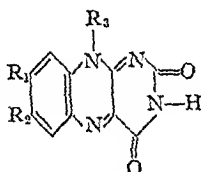
dichloro compound (Ia) has been reported by Barlow (3) as an intermediate in his synthesis of 6,7-dichloro-9-(2'-hydroxyethyl)-isoalloxazine. However, he did not isolate the compound. Evaporation of an alcoholic solution of the compound gave a product which could be purified and a correct analysis for carbon-hydrogen was obtained. A method



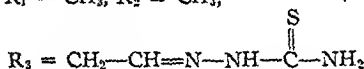
- Ia  $R_1 = \text{Cl}, R_2 = \text{Cl}$   
 Ib  $R_1 = \text{CH}_3, R_2 = \text{CH}_3$

for the preparation of the dimethyl compound (Ib) involved the reaction of 4-chloro-5-nitro-*o*-xylene with 2-aminoethanol followed by hydrogenation.

The two corresponding isoalloxazines and a thiosemicarbazone derivative (IIa, IIb, and IIc) were prepared to determine the influence of the sugar



- IIa  $R_1 = \text{Cl}, R_2 = \text{Cl}, R_3 = \text{CH}_2\text{--CH}_2\text{OH}$   
 IIb  $R_1 = \text{CH}_3, R_2 = \text{CH}_3, R_3 = \text{CH}_2\text{--CH}_2\text{OH}$   
 IIc  $R_1 = \text{CH}_3, R_2 = \text{CH}_3,$



group found in the isoalloxazines previously described (7). Compound IIa had been prepared previously by Barlow (3) from Ia and alloxan. Compound IIb was prepared by cleavage of the sugar portion of riboflavin as reported by Fall and Pterling (2). The thiosemicarbazone derivative (IIc) was also prepared by the method of Fall and Pterling.

**Chelate Formation.**—Initially the preparation of the metal chelates was attempted in a manner similar to the preparation of metal chelates of riboflavin (8). However, the isoalloxazines required a pH of 12 for them to remain in solution in water. At this pH the major product obtained was the metal hydroxides. In order to prevent the formation of metal hydroxides, the solvent was changed to ethylene glycol and the pH adjusted to 9. Aqueous solutions of various metal salts were then added to the isoalloxazine solutions in equimolar quantities. A marked drop in pH which occurred in the absence of added alkali and immediate precipitation provides evidence for chelation rather than salt formation. Further evidence was provided by the absence of metal ions in the supernatant solutions as indicated by tests with ferricyanide. Unlike the riboflavin chelates (8) only chelates of copper could be obtained in a pure state. Thus, the sugar side chain of riboflavin must be involved in chelate formation of the hexacoordinate metals: namely, cobalt, nickel, and iron. This is not in agreement with the postulation of Albert (9) that riboflavin should chelate like 8-hydroxyquinoline. If this

were true the sugar group would not be involved in chelation with any metal.

The chelates of the 4,5-dichloro and 4,5-dimethyl-N-(2'-hydroxyethyl)-anilines were prepared in an ethanol-water mixture, due to the insolubility of the parent compounds in water, by the same procedure as described above. This procedure using cupric chloride gave immediate precipitation of a dark purple chelate with the dichloro compound and a pale green chelate with the dimethyl compound. As before only the copper chelate could be isolated in a pure state. Various other metals were attempted: namely, cobalt, nickel, iron ( $\text{Fe}^{++}$ ), and zinc.

**Structural Considerations.**—The results of elemental analyses of the chelates, as shown in Table I, indicate a structure containing one metal atom per molecule of isoalloxazine or substituted aniline. The presence of water of chelation was shown by a loss of weight corresponding to approximately the calculated number of molecules of water without a change in color when the chelates were dehydrated at 250°. Only in the case of the copper chelate of IIb was any color change noted during the dehydration procedure. It darkened and appeared to decompose. In the case of the thiosemicarbazone derivative (IIc) the elemental analyses indicated a structure closely related to riboflavin (8) containing two metal atoms per molecule of isoalloxazine.

The ultraviolet absorption spectral characteristics of the isoalloxazines and their copper derivatives were compared in an attempt to show differences between the chelate and the parent compound. Little change in the position of the maximum absorption for the isoalloxazines occurred on formation of the chelates, as was previously observed for the chelates of riboflavin (10). The position of maximum absorption for the aniline compounds (Ia and Ib) shifted from 253  $\text{m}\mu$  to 240  $\text{m}\mu$ . No attempt at an explanation of this shift will be presented at this time.

**Biological Results.**—As shown in Table II, the administration of nondiabetogenic doses of the chelates produced a variety of responses in rabbits. The dichloro compounds, as chelates, produced increases in the blood sugar level far greater than did alloxan. The differences between the blood sugar increases caused by the chelates and the nonmetallized agents is striking. The dichloro compounds also were more toxic than alloxan. A dose greater than 50 mg./Kg. in most cases was fatal to the rabbits. The dimethyl compounds, both chelated and unchelated, in general were inactive. Riboflavin containing a sugar side chain produced a slightly greater blood sugar response as the chelate than alloxan. In the case where the sugar group of riboflavin was replaced by a thiosemicarbazone, or a hydroxyethyl group, neither the chelate nor the parent compound showed any appreciable effect.

These observations suggest that in some way, either chloro substitution or the presence of a sugar side chain on the isoalloxazine molecule are contributing groups in the production of blood sugar increases caused by the chelates of these compounds. Chelation alone is apparently not the final answer to the activity of various isoalloxazines, fragments of isoalloxazines, or of alloxan.

TABLE I.—ANALYSES OF THE METAL CHELATES<sup>a</sup>

Chelate	Formula	C, %	H, %	Cu, %	H <sub>2</sub> O, <sup>b</sup> %	Yield	Color
6,7-Dimethyl-9-(2'-hydroxyethyl)-isoalloxazine, Cu	C <sub>14</sub> H <sub>12</sub> N <sub>4</sub> O <sub>3</sub> Cu + OH <sup>-</sup> · H <sub>2</sub> O	Calcd. 41.83 Found 42.64	4.51 5.67	15.80 12.94	4.8 ...	87	Brown
6,7-Dichloro-9-(2'-hydroxyethyl)-isoalloxazine, Cu	C <sub>12</sub> H <sub>6</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub> Cu + OH <sup>-</sup> · H <sub>2</sub> O	Calcd. 33.93 Found 33.60	2.38 3.49	14.96 14.14	4.3 4.4	80	Green
Thiosemicarbazone of 6,7-dimethyl-9-formylmethyl-isoalloxazine, Cu <sub>2</sub>	C <sub>16</sub> H <sub>11</sub> N <sub>7</sub> O <sub>2</sub> SCu <sub>2</sub> · 4H <sub>2</sub> O	Calcd. 32.60 Found 32.26	3.46 5.07	23.0 21.8	13.0 12.2	50	Green
2-Amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline, Cu	C <sub>16</sub> H <sub>16</sub> N <sub>2</sub> OCu + Cl <sup>-</sup> · H <sub>2</sub> O	Calcd. 40.55 Found 40.00	5.78 4.43	21.45 22.80	6.1 5.8	34	Green
2-Amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline, Cu	C <sub>9</sub> H <sub>9</sub> Cl <sub>2</sub> N <sub>2</sub> OCu + Cl <sup>-</sup> · H <sub>2</sub> O	Calcd. 28.79 Found 28.87	3.19 1.94	18.86 18.60	5.4 6.5	73	Purple

<sup>a</sup> The carbon-hydrogen analyses were determined by Weiler and Strauss, Oxford, England. The copper analyses were conducted by a standard ashing procedure. The chelates were dried *in vacuo* at 100° before analysis for carbon and hydrogen.

<sup>b</sup> This quantity is the loss of weight on dehydration at 250° for two hours.

<sup>c</sup> This compound gradually darkened during dehydration.

TABLE II.—BLOOD SUGAR DETERMINATIONS OF METAL CHELATES IN RABBITS<sup>a</sup>

Compound	Increase in Blood Sugar Level, mg. %			
	1/2	1	2	3
Alloxan <sup>b</sup>	15	48	4	...
6,7-Dimethyl-9-(1-D-ribityl)-II, Co <sup>b</sup>	23	72	50	-5
6,7-Dichloro-9-(1-D-sorbityl)-II, Co <sup>b</sup>	60	110	130	125
6,7-Dimethyl-9-(2'-hydroxyethyl)-II, Cu	0	-15	-10	...
6,7-Dichloro-9-(2'-hydroxyethyl)-II, Cu	57	85	80	37
6,7-Dimethyl-9-formylmethyl-II-thiosemicarbazone, Cu <sub>2</sub>	0	0	-3	.
2-Amino-4,5-dimethyl-N-(2'-hydroxyethyl)-I, Cu	0	-7	-11	-13
2-Amino-4,5-dichloro-N-(2'-hydroxyethyl)-I, Cu	70	75	100	30

<sup>a</sup> The mode of administration in all cases was intraperitoneal, and the dosage 20 mg./Kg. In all cases the non-chelated compounds produced no change in the blood sugar level in rabbits.

<sup>b</sup> Inserted as comparison compounds (7).

<sup>c</sup> I = aniline, II = isoalloxazine.

## EXPERIMENTAL

The melting points were taken on a Fisher-Johns block and are recorded as read. The elemental analyses were carried out in the Weiler and Strauss Microanalytical Laboratory, Oxford, England.

**2-Amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline.**—A solution of 5.0 Gm. (0.02 mole) of 2-nitro-4,5-dichloro-N-(2'-hydroxyethyl)-aniline (3) and 150 ml. of ethanol was obtained with the aid of heat. One-tenth gram of platinum oxide (Adam's catalyst, American Platinum Works) was added to the alcoholic solution and the solution was reduced in a Paar hydrogenator at 40 p. s. i. The reduction required about thirty minutes. The catalyst was removed by filtration and the alcohol was removed

from the filtrate by vacuum distillation. Recrystallization of the product from water produced 3.5 Gm. (80% yield) of 2-amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline as purple needles, m. p. 128.5–130°.

*Anal.*—Calcd. for C<sub>9</sub>H<sub>9</sub>Cl<sub>2</sub>N<sub>2</sub>O: C, 43.43; H, 4.55. Found: C, 43.51; H, 4.68.

**2-Nitro-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline.**—A solution of 3.0 Gm. (0.01 mole) of 4-chloro-5-nitro-*o*-xylene (11) which contained an excess of 2-aminoethanol (5.0 Gm.) was refluxed in 100 ml. of pyridine for seventy-two hours. The pyridine was removed by heating under reduced pressure and the resulting solid was recrystallized from an ethanol:water:methanol mixture (4:4:2). A yield of 1.7 Gm. (52%) of 2-nitro-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline was obtained as red needles which melted at 195–197°.

*Anal.*—Calcd. for C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>: C, 57.14; H, 6.71. Found: C, 56.86; H, 6.66.

**2-Amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline.**—2-Nitro-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline was reduced in the manner described for 2-amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline. Recrystallization of the product from a 50% ethanol : water mixture gave a 66% yield of 2-amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline as purple platelets; m. p. 160° (decomp.).

*Anal.*—Calcd. for C<sub>10</sub>H<sub>16</sub>N<sub>2</sub>O: C, 66.64; H, 8.95. Found: C, 67.28; H, 9.06.

**6,7-Dimethyl-9-(2'-hydroxyethyl)-isoalloxazine and Thiosemicarbazone.**—These compounds were prepared from 6,7-dimethyl-9-formylmethylisoalloxazine hydrate according to the procedure reported by Fall and Petering (2).

**6,7-Dichloro-9-(2'-hydroxyethyl)-isoalloxazine.**—This compound was prepared by modification of the methods of Barlow (3) and Holly, *et al.* (12). A hydrogenated solution of 12.8 Gm. (0.05 mole) of 2-nitro-4,5-dichloro-N-(2'-hydroxyethyl)-aniline (3) was filtered into a suspension of 14.8 Gm. (0.09 mole) of alloxan monohydrate and 33.6 Gm. (0.04 mole) of boric acid in 600 ml. of

acetic acid. The mixture was stirred intermittently at room temperature for three days and then filtered. The filtrate was evaporated to dryness *in vacuo*. The solid material obtained upon filtration and evaporation was suspended in boiling water to remove the boric acid. The solid remaining was collected and recrystallized from ethylene glycol. The green amorphous solid was washed with water and dried *in vacuo*. The yield was 11.9 Gm (65%).

**Preparation of the Metal Chelates.**—The copper chelates of 6,7-dichloro, and 6,7-dimethyl-9-(2'-hydroxyethyl)-isoalloxazine as well as the thiosemicarbazone derivative were prepared by the same procedure.

To a mixture of the isoalloxazine (0.03 mole) in hot ethylene glycol was added sufficient 10% sodium hydroxide solution to dissolve the isoalloxazine. An aqueous solution of anhydrous cupric chloride (0.03 mole) was added slowly to the stirred solution along with sufficient 10% sodium hydroxide solution to maintain a pH of 9. After thirty minutes of further stirring, the solid which formed was collected and washed with water and acetone. Analyses, yields, and colors of the various chelates are shown in Table I.

The copper chelates of 2-amino-4,5-dichloro and 2-amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline were prepared by dissolving 0.005 mole of each in 100 ml. of a 50% ethanol:water mixture. Sufficient 10% sodium hydroxide solution was added to bring the pH to 9. An aqueous solution of 0.85 Gm (0.005 mole) of anhydrous cupric chloride was then added slowly over a period of ten minutes, along with sufficient 10% sodium hydroxide solution to maintain the pH at 9. After one hour of stirring,

the product was isolated and purified by washing with water and acetone. Analyses, yields, and colors of the various chelates are shown in Table I. In no case did any of the chelates melt under 300°.

**Determination of Blood Sugar Levels.**—Normal blood sugar levels were determined in rabbits fasted for twelve hours prior to injection of the chelates or parent compounds. The sample for injection was passed through a No. 100 sieve and suspended in 3 ml. of water with the aid of a small quantity of sodium lauryl sulfate. The chelates were injected by the intraperitoneal route due to their poor solubility in water. After injection, blood samples were taken at one-half- to one-hour intervals, and the sugar content determined by the standard Micro-Folin-Wu method using a Bausch and Lomb "Spectronic 20" spectrophotometer.

## REFERENCES

- (1) Kuhn, R., and Weygand, F., *Ber.*, 67B, 2084(1934)
- (2) Fall, H. H., and Petering, H. G., *J. Am. Chem. Soc.*, 78, 377(1956)
- (3) Barlow, R. B., *J. Chem. Soc.*, 1951, 2225
- (4) Bardos, T. J., Olsen, D. B., and Enkoji, T., *J. Am. Chem. Soc.*, 79, 4703(1957)
- (5) Binet, L., Wellers, G., and Marquis, M., *Compt. rend.*, 229, 1185(1949)
- (6) Bruckmann, G., and Werthheimer, E., *J. Biol. Chem.*, 168, 241(1947)
- (7) Lange, W. E., and Foye, W. O., *This Journal*, 45, 699(1956)
- (8) Foye, W. O., and Lange, W. E., *J. Am. Chem. Soc.*, 76, 2199(1956)
- (9) Albert, A., *Biochem. J.*, 54, 646(1953)
- (10) Mahler, H. R., Fairhurst, A. S., and Mackler, B., *J. Am. Chem. Soc.*, 77, 1514(1955)
- (11) Adams, R. R., Weisel, C. A., and Mosher, H. S., *ibid.*, 68, 883(1946)
- (12) Holly, F. W., Peel, E. W., Mozingo, R., and Folkers, K., *ibid.*, 72, 5416(1950)

## Determination of the Effect of the Hydrophil-Lipophil Balance on Drug Release from Bases by an *In Vitro* Colorimetric Method\*

By ROGER Y. SPITTLE and CHARLES W. HARTMAN

Semisolid bases were prepared from liquid petrolatum and various emulsifying agents were added to produce a specific hydrophil-lipophil balance in the bases. A water-soluble dye was added to each base and its release into water was measured by a colorimetric method. The data obtained indicates that the HLB value of a base influences the rate of drug release from the base. The importance of surface area and drug concentration is demonstrated and other factors that probably influence drug release are suggested.

MANY INVESTIGATIONS have been conducted concerning drug release from ointment and suppository bases and the effect of the base on the therapeutic action of the ointment or suppository

(1-9). The incorporation of emulsifying agents into ointment bases has been shown to have a definite influence on drug release (5, 7, 10, 11).

Hartman and LaRocca (6) found, using a colorimetric test for dye release from suppository bases, that emulsifying agents slightly increased the dye release. This was demonstrated by Whitworth (7) using a colorimetric method in

\* Received August 21, 1959, from the University of Georgia, School of Pharmacy, Athens.  
Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.

TABLE I—ANALYSES OF THE METAL CHELATES<sup>a</sup>

Chelate	Formula		C, %	II, %	Cu, %	H <sub>2</sub> O <sup>b</sup> %	Yield	Color
6,7-Dimethyl-9-(2'-hydroxyethyl)-isoalloxazine, Cu	C <sub>14</sub> H <sub>12</sub> N <sub>4</sub> O <sub>3</sub> Cu + OH <sup>-</sup> · H <sub>2</sub> O	Calcd	41 83	4 51	15 80	4 8		
		Found	42 64	5 67	12 94	<sup>c</sup>	87	Brown
6,7-Dichloro-9-(2'-hydroxyethyl)-isoalloxazine, Cu	C <sub>12</sub> H <sub>6</sub> Cl <sub>2</sub> N <sub>4</sub> O <sub>3</sub> Cu + OH <sup>-</sup> · H <sub>2</sub> O	Calcd	33 93	2 38	14 96	4 3		
		Found	33 60	3 49	14 14	4 4	80	Green
Thiosemicarbazone of 6,7-dimethyl-9-formylmethyl-isoalloxazine, Cu <sub>2</sub>	C <sub>15</sub> H <sub>11</sub> N <sub>7</sub> O <sub>2</sub> SCu <sub>2</sub> 4H <sub>2</sub> O	Calcd	32 60	3 46	23 0	13 0		
		Found	32 26	5 07	21 8	12 2	50	Green
2-Amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline, Cu	C <sub>16</sub> H <sub>16</sub> N <sub>2</sub> OCu + Cl <sup>-</sup> · H <sub>2</sub> O	Calcd	40 55	5 78	21 45	6 1		
		Found	40 00	4 43	22 80	5 8	34	Green
2-Amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline, Cu	C <sub>9</sub> H <sub>6</sub> Cl <sub>2</sub> N <sub>2</sub> OCu + Cl <sup>-</sup> · H <sub>2</sub> O	Calcd	28 79	3 19	18 86	5 4		
		Found	28 87	1 94	18 60	6 5	73	Purple

<sup>a</sup> The carbon-hydrogen analyses were determined by Weiler and Strauss, Oxford, England. The copper analyses were conducted by a standard ashing procedure. The chelates were dried *in vacuo* at 100° before analysis for carbon and hydrogen.

<sup>b</sup> This quantity is the loss of weight on dehydration at 250° for two hours.

<sup>c</sup> This compound gradually darkened during dehydration.

TABLE II—BLOOD SUGAR DETERMINATIONS OF METAL CHELATES IN RABBITS<sup>a</sup>

Compound	Increase in Blood Sugar Level, mg %			
	1/2	1	2	3
Alloxan <sup>b</sup>	15	48		
6,7-Dimethyl-9-(1-D-ribityl)-II, Co <sup>b</sup>	23	72	50	-5
6,7-Dichloro-9-(1-D-sorbitol)-II, Co <sup>b</sup>	60	110	130	125
6,7-Dimethyl-9-(2'-hydroxyethyl)-II, Cu	0	-15	-10	
6,7-Dichloro-9-(2'-hydroxyethyl)-II, Cu	57	85	80	37
6,7-Dimethyl-9-formylmethyl-II-thiosemicarbazone, Cu <sub>2</sub>	0	0	-3	
2-Amino-4,5-dimethyl-N-(2'-hydroxyethyl)-I, Cu	0	-7	-11	-13
2-Amino-4,5-dichloro-N-(2'-hydroxyethyl)-I, Cu	70	75	100	30

<sup>a</sup> The mode of administration in all cases was intraperitoneal, and the dosage 20 mg/Kg. In all cases the non-chelated compounds produced no change in the blood sugar level in rabbits.

<sup>b</sup> Inserted as comparison compounds (7).

<sup>c</sup> I = aniline, II = isoalloxazine.

## EXPERIMENTAL

The melting points were taken on a Fisher-Johns block and are recorded as read. The elemental analyses were carried out at the Weiler and Strauss Microanalytical Laboratory, Oxford, England.

**2-Amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline.**—A solution of 5.0 Gm (0.02 mole) of 2-nitro-4,5-dichloro-N-(2'-hydroxyethyl)-aniline (3) and 150 ml of ethanol was obtained with the aid of heat. One-tenth gram of platinum oxide (Adam's catalyst, American Platinum Works) was added to the alcoholic solution and the solution was reduced in a Paar hydrogenator at 40 p s i. The reduction required about thirty minutes. The catalyst was removed by filtration and the alcohol was removed

from the filtrate by vacuum distillation. Recrystallization of the product from water produced 3.5 Gm (80% yield) of 2-amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline as purple needles, m p 128.5–130°.

**Anal.**—Calcd for C<sub>8</sub>H<sub>10</sub>Cl<sub>2</sub>N<sub>2</sub>O: C, 43.43; H, 4.55. Found: C, 43.51; H, 4.68.

**2-Nitro-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline.**—A solution of 3.0 Gm (0.01 mole) of 4-chloro-5-nitro-*o*-xylene (11) which contained an excess of 2-aminoethanol (5.0 Gm.) was refluxed in 100 ml. of pyridine for seventy-two hours. The pyridine was removed by heating under reduced pressure and the resulting solid was recrystallized from an ethanol-water-methanol mixture (4:4:2). A yield of 1.7 Gm (52%) of 2-nitro-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline was obtained as red needles which melted at 195–197°.

**Anal.**—Calcd for C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>O<sub>3</sub>: C, 57.14; H, 6.71. Found: C, 56.86; H, 6.66.

**2-Amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline.**—2-Nitro-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline was reduced in the manner described for 2-amino-4,5-dichloro-N-(2'-hydroxyethyl)-aniline. Recrystallization of the product from a 50% ethanol:water mixture gave a 66% yield of 2-amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline as purple platelets; m. p. 160° (decolor).

**Anal.**—Calcd for C<sub>10</sub>H<sub>16</sub>N<sub>2</sub>O: C, 66.64; H, 8.95. Found: C, 67.28; H, 9.06.

**6,7-Dimethyl-9-(2'-hydroxyethyl)-isoalloxazine and Thiosemicarbazone.**—These compounds were prepared from 6,7-dimethyl-9-formylmethylisoalloxazine hydrate according to the procedure reported by Fall and Petering (2).

**6,7-Dichloro-9-(2'-hydroxyethyl)-isoalloxazine.**—This compound was prepared by modification of the methods of Barlow (3) and Holly, *et al.* (12). A hydrogenated solution of 12.8 Gm (0.05 mole) of 2-nitro-4,5-dichloro-N-(2'-hydroxyethyl)-aniline (3) was filtered into a suspension of 14.8 Gm (0.09 mole) of alloxan monohydrate and 33.6 Gm (0.04 mole) of boric acid in 600 ml of

acetic acid. The mixture was stirred intermittently at room temperature for three days and then filtered. The filtrate was evaporated to dryness *in vacuo*. The solid material obtained upon filtration and evaporation was suspended in boiling water to remove the boric acid. The solid remaining was collected and recrystallized from ethylene glycol. The green amorphous solid was washed with water and dried *in vacuo*. The yield was 11.9 Gm. (65%).

**Preparation of the Metal Chelates.**—The copper chelates of 6,7-dichloro, and 6,7-dimethyl-9-(2'-hydroxyethyl)-isoalloxazine as well as the thio-semicarbazone derivative were prepared by the same procedure.

To a mixture of the isoalloxazine (0.03 mole) in hot ethylene glycol was added sufficient 10% sodium hydroxide solution to dissolve the isoalloxazine. An aqueous solution of anhydrous cupric chloride (0.03 mole) was added slowly to the stirred solution along with sufficient 10% sodium hydroxide solution to maintain a pH of 9. After thirty minutes of further stirring, the solid which formed was collected and washed with water and acetone. Analyses, yields, and colors of the various chelates are shown in Table I.

The copper chelates of 2-amino-4,5-dichloro and 2-amino-4,5-dimethyl-N-(2'-hydroxyethyl)-aniline were prepared by dissolving 0.005 mole of each in 100 ml of a 50% ethanol-water mixture. Sufficient 10% sodium hydroxide solution was added to bring the pH to 9. An aqueous solution of 0.85 Gm (0.005 mole) of anhydrous cupric chloride was then added slowly over a period of ten minutes, along with sufficient 10% sodium hydroxide solution to maintain the pH at 9. After one hour of stirring,

the product was isolated and purified by washing with water and acetone. Analyses, yields, and colors of the various chelates are shown in Table I. In no case did any of the chelates melt under 300°.

**Determination of Blood Sugar Levels.**—Normal blood sugar levels were determined in rabbits fasted for twelve hours prior to injection of the chelates or parent compounds. The sample for injection was passed through a No. 100 sieve and suspended in 3 ml. of water with the aid of a small quantity of sodium lauryl sulfate. The chelates were injected by the intraperitoneal route due to their poor solubility in water. After injection, blood samples were taken at one-half- to one-hour intervals, and the sugar content determined by the standard Micro-Folin-Wu method using a Bausch and Lomb "Spectronic 20" spectrophotometer.

## REFERENCES

- (1) Kuhn, R., and Weygand, F., *Ber.*, 67B, 2084 (1934)
- (2) Fall, H. H., and Petering, H. G., *J. Am. Chem. Soc.*, 78, 377 (1956)
- (3) Barlow, R. B., *J. Chem. Soc.*, 1951, 2225
- (4) Bardos, T. J., Olsen, D. B., and Enkoji, T., *J. Am. Chem. Soc.*, 79, 4704 (1957)
- (5) Binet, L., Wellers, G., and Marquis, M., *Compt. rend.*, 229, 1185 (1949)
- (6) Bruckmann, G., and Werthheimer, E., *J. Biol. Chem.*, 168, 241 (1947)
- (7) Lange, W. E., and Foye, W. O., *THIS JOURNAL*, 45, 699 (1956)
- (8) Foye, W. O., and Lange, W. E., *J. Am. Chem. Soc.*, 76, 2199 (1956)
- (9) Albert, A., *Biochem. J.*, 54, 646 (1953)
- (10) Mahler, H. R., Fairhurst, A. S., and Mackler, B., *J. Am. Chem. Soc.*, 77, 1514 (1955)
- (11) Adams, R. R., Weisel, C. A., and Mosher, H. S., *ibid.*, 68, 883 (1946)
- (12) Holly, F. W., Peel, E. W., Mazingo, R., and Folkers, K., *ibid.*, 72, 5416 (1950)

# Determination of the Effect of the Hydrophil-Lipophil Balance on Drug Release from Bases by an *In Vitro* Colorimetric Method\*

By ROGER Y. SPITTLE and CHARLES W. HARTMAN

Semisolid bases were prepared from liquid petrolatum and paraffin. Various emulsifying agents were added to produce a specific hydrophil-lipophil balance in the bases. A water-soluble dye was added to each base and its rate of diffusion into water was measured by a colorimetric method. The data obtained indicate that the HLB value of a base influences the rate of drug release from the base. The importance of surface area and drug concentration is demonstrated and other factors that probably influence drug release are suggested.

**M**ANY INVESTIGATIONS have been conducted concerning drug release from ointment and suppository bases and the effect of the base on the therapeutic action of the ointment or suppository

(1-9). The incorporation of emulsifying agents into ointment bases has been shown to have a definite influence on drug release (5, 7, 10, 11).

Hartman and LaRocca (6) found, using a colorimetric test for dye release from suppository bases, that emulsifying agents slightly increased the dye release. This was demonstrated by Whitworth (7) using a colorimetric method in

\* Received August 21, 1959, from the University of Georgia, School of Pharmacy, Athens.  
Presented to the Scientific Section, A. Ph. A., Cincinnati Meeting, August 1959.



conjunction with an *in vivo* study of suppositories with rabbits.

Rhyné and co-workers (5), using a bacteriological assay method, found that various HLB in factors ointment bases gave different degrees of inhibition activity. This variation of inhibition with HLB was also found to be dependent on the physico-chemical properties of the antibiotic or antiseptic used.

A variation of HLB value within an ointment or suppository base should, upon administration, give a corresponding variation in the release of medicament from the base. At least part of the base should be emulsified with tissue fluids and cause a greater absorption of the drug.

The effects of nonionic emulsifying agents on the release of medication from hydrophillic ointment bases were tested by the agar plate method (12) and optimum release of medicament was obtained when the surfactant was employed in a 1 per cent concentration. These authors concluded that an increase in the surfactant concentration caused a decrease in the release of medication.

The purpose of this experiment was to determine, by an *in vitro* colorimetric method, the effect of the HLB on the release of a water-soluble dye from various bases. These bases were composed of mineral oil; paraffin; and 10 per cent, by weight, of several emulsifying agents having different HLB values. No attempt has been made to prepare ointment or suppository bases with physical properties or appearance acceptable for commercial use. However, efforts have been made to control those properties having an influence on drug release. The consistency of the bases was similar to the consistency of suppositories because it was hoped to compare work in the present studies with that from *in vivo* studies of suppositories using the same base.

## EXPERIMENTAL

**Preparation of Bases.**—The bases were composed of liquid petrolatum; paraffin; and 10%, by weight, of emulsifying agents. The emulsifying agents were the same as those used by Rhyné and Hartman (5) with the HLB values varying from 1.8 to 17.9. The consistency of the bases was adjusted by varying the per cent of paraffin and liquid petrolatum. All bases were prepared by fusion.

Two per cent amaranth water-soluble dye (FD&C Red No. 2) was added to the various bases by fusion. As shown in Fig. 2 this concentration showed a gradual release of the dye over a fifteen-minute period.

**Colorimetric Method of Measuring Dye Release from Bases.**—Fifteen milligrams of the base containing the dye was accurately weighed using an analytical balance and spread in a circle 1.0 cm. in diameter on 1.5 by 3.5 cm. strips of Whatman filter

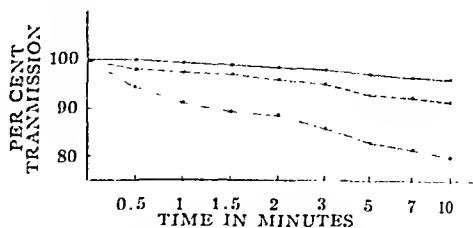


Fig. 1.—Per cent transmission of dye solution after release of dye from a base having an HLB value of 17.9, spread on different surface areas. Each base contained 10% of the emulsifying agent. — 0.4 cm. diameter, - - - 0.6 cm. diameter, - . . . 1.0 cm. diameter.

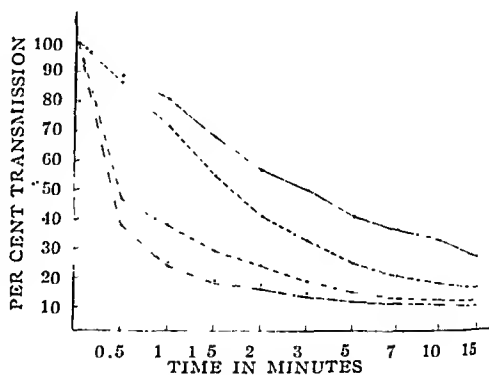


Fig. 2.—Per cent transmission of dye solutions after release of dye from a base with an HLB value of 10.5 containing different concentrations of dye. — 2% dye, - - - 4% dye, - . . . 6% dye, . . . . 8% dye, - - - - 10% dye.

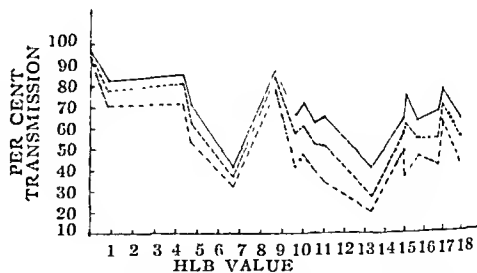


Fig. 3.—Per cent transmission of dye solutions after release of dye from bases having different HLB values. — Per cent transmission after three minutes, - - - per cent transmission after five minutes, - . . . per cent transmission after ten minutes.

paper, No. 5. As shown in Fig. 1 the best ointment base diameter for the analytical procedure was 1.0 cm. By use of a No. 5 cork holding a small looped wire, the paper could be suspended in 18 cc. of water in a 19 by 105 mm. colorimeter tube. The paper did not contact the sides of the tube or the stopper, and was suspended slightly above the light path. The determination of the release of the dye from the bases was accomplished by measuring the per cent transmission of the solution on a Coleman junior spectrophotometer, model 6A. The per cent transmission readings on the colorimeter were made at definite time intervals after the base, on the paper

TABLE I.—PER CENT TRANSMISSION OF SOLUTIONS OF DYE RELEASED FROM BASES WITH AN HLB VALUE OF 17.9<sup>a</sup>

Emulsifying Agent, %	Time in Minutes								
	0.5	1	1.5	2	3	5	7	10	15
1	99.4	98.6	98.6	98.6	98.4	98.0	97.4	96.5	95.6
10	98.4	97.0	96.5	96.0	95.0	93.4	92.6	92.0	91.0
20	97.5	96.4	95.6	95.4	94.6	93.0	92.6	92.0	91.0
30	97.0	96.5	96.0	95.4	94.6	93.6	92.6	91.6	91.5
40	97.0	96.4	95.5	95.5	94.4	93.4	92.0	90.4	87.4
50	96.6	96.0	95.4	94.6	94.0	92.0	89.6	87.6	84.5

<sup>a</sup> Surface area 10 cm<sup>2</sup>, 2% dye

strip, came into contact with the distilled water in the colorimeter tubes. The time intervals at which readings were made are shown in Figs. 1, 2, and 3. The water had been placed in the colorimeter tubes prior to immersing the paper strips and was maintained at a temperature of 30° in a constant temperature bath until readings on the colorimeter were begun. A control base consisting of petrolatum only and 2% dye was treated in exactly the same manner. This control is recorded as an HLB base with a value of 0.

Figure 3 shows the per cent transmission of the dye solutions at three, five, and ten-minute intervals for the bases tested.

To determine the effect of a change in the concentration of the emulsifying agent bases were prepared containing 1, 10, 20, 30, 40, and 50% emulsifying agent with an HLB value of 17.9 and containing 2% dye. The results are given in Table I.

## DISCUSSION OF RESULTS

**Effect of HLB on Dye Release.**—Results of the present work indicate a definite effect of the HLB factor on the release of medicament from bases. As shown in Fig. 3, with a variation of the HLB value, a change in the rate of release of the water-soluble dye was exhibited. The greatest rate of release of dye was observed at an HLB of 6.7 and 13.3. In general, the bases with lower HLB values gave a lesser rate of dye release than those with higher HLB values.

Petrolatum bases with no emulsifying agent gave very little dye release. This seems logical since their HLB values would be near zero.

The results found by the present colorimetric method using a water-soluble dye are in partial agreement with those found by Rhyne and Hartman (5). In their bacterial inhibition studies of ointment bases having different HLB values they found that neomycin sulfate, a water-soluble antibiotic, showed greater inhibition of bacteria in the bases with higher HLB values.

It is possible that several factors play an important part in the effect the HLB value has on drug release from a base. Three of these may be: the emulsification of the base with the body fluids, the distribution coefficient of the drug between the base and body fluids, and the intermolecular reaction between the drug and the surfactant.

Further studies using dyes less soluble in water should show, to some extent, the effect of drug solubility on release.

## CONCLUSIONS

1 The HLB value of a base has a definite influence on the rate of release of medicament from the base.

2 The surface area of the base is a significant factor in the rate of drug release from bases.

3 The concentration of the drug in the base is of prime importance in the rate of release of the medicament from the base.

4 An increase in the concentration of the emulsifying agent in the base increases the rate of release of a water-soluble drug.

## REFERENCES

- (1) Reddish, G. F., and Wales, H. J., *THIS JOURNAL*, **18**, 576 (1929).
- (2) Johnston, G. W., and Lee, C. O., *ibid.*, **32**, 278 (1943).
- (3) Foley, E., and Lee, C. O., *ibid.*, **31**, 105 (1942).
- (4) Bliss, A. R., Jr., *J. Tenn. Acad. Sci.*, **11**, 83 (1936).
- (5) Rhyne, J. W., Payne, W. J., and Hartman, C. W., *THIS JOURNAL*, **49**, 234 (1960).
- (6) Hartman, C. W., and LaRocca, J. P., *THIS JOURNAL*, **45**, 86 (1956).
- (7) Whitworth, C. W., and LaRocca, J. P., *ibid.*, **48**, 353 (1959).
- (8) Gross, H. M., and Becker, C. H., *ibid.*, **42**, 90 (1953).
- (9) Neuroth, M. L., and Lee, C. O., *ibid.*, **6**, 285 (1945).
- (10) Dodd, M. C., Hartmann, F. W., and Ward, W. C., *ibid.*, **35**, 33 (1946).
- (11) McDonald, L. H., and Himehick, R. E., *ibid.*, **37**, 368 (1948).
- (12) Barker, D. Y., DeKay, H. G., and Christian, J. E., *ibid.*, **45**, 527 (1956).

# Steroids I\*

## Synthesis of Steroid Nitrogen Mustards

By ROBERT E. HAVRANEK† and NORMAN J. DOORENBOS

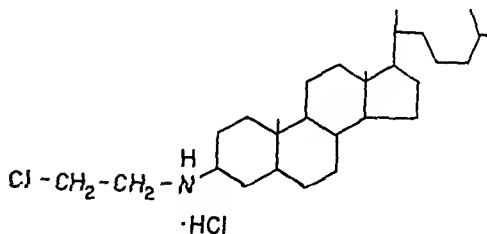
The synthesis of the steroid nitrogen mustards, 3 $\alpha$ -(2-chloroethylamino)cholestane hydrochloride and 3 $\beta$ -(2-chloroethylamino)cholestane hydrochloride, and their intermediates, 3 $\alpha$ -(2-hydroxyethylamino)cholestane and 3 $\beta$ -(2-hydroxyethylamino)cholestane are described. These compounds were inactive in tumor tests administered by the National Service Center for Cancer Chemotherapy.

CANCER, which killed 255,000 Americans in 1958, is receiving more attention today than probably any other disease. Chemotherapy has been a useful tool for the treatment of patients with inoperable cancer. The most useful drugs have been metabolic antagonists, alkylating agents, and steroids.

In view of these facts, the synthesis of steroid alkylating agents for evaluation as cancer chemotherapeutic agents became of interest. A search of the literature revealed that some steroid nitrogen mustard alkylating agents had been synthesized. These compounds included N,N-bis(2-chloroethyl)-3-amino-5-cholestene hydrochloride (1), N,N-bis(2-chloroethyl)-3-ergosterylamine hydrochloride (2), N,N-bis(2-chloroethyl)-3-stigmasterylamine hydrochloride (3), and 3 $\beta$ -[bis(2-chloroethyl)aminoethyl]-5-cholestene and its hydrochloride (4). Biological data were reported only on the last of these compounds. Toxicity studies of this compound were inconclusive because of its low solubility.

Formerly it was thought that a nitrogen mustard must have at least two reactive moieties per molecule for activity (5, 6). It was postulated that the nitrogen mustard must be capable of forming a cross link between two essential protein molecules, or between two portions of the same molecule. Recent facts, however, have demonstrated that monofunctional nitrogen mustards are useful in the treatment of cancer, al-

though they are not nearly as active. Bond (7) has stated that the monofunctional nitrogen mustards should receive further study since they may have a better therapeutic index.



3 $\beta$ -(2-Chloroethylamino)cholestane Hydrochloride

The synthesis of nitrogen mustard derivatives of the 3-aminocholestanes with one reactive moiety per molecule was chosen for this investigation.

The 3-aminocholestanes were prepared from 3-cholestanone oxime using the stereospecific methods of reduction developed by Shoppee. Reduction with sodium and ethanol yielded 3 $\beta$ -aminocholestane (8, 9). Reduction by hydrogen in glacial acetic acid with a platinum catalyst yielded 3 $\alpha$ -aminocholestane (10).

Pure samples of these amines were obtained only in small yield after long purification procedures including recrystallization and chromatography. Shoppee had similar difficulties (11). Probably these amines form intermolecular complexes with steroid impurities which are difficult to separate. Such complexes have been observed frequently with steroid alcohols. For these reasons, the impure amines were used in the next synthetic step. The products proved to be much easier to purify.

3 $\beta$ -Aminocholestane was condensed with 2-chloroethanol in the presence of sodium carbonate to yield 3 $\beta$ -(2-hydroxyethylamino)cholestane. 3 $\alpha$ -(2-Hydroxyethylamino)cholestane was prepared by condensation with the more reactive reagent, 2-bromoethanol. The nitrogen mustard derivatives were prepared by the reaction of thionyl chloride with these alcohols. Infrared spectra were used as an aid in the identification of these compounds.

3-Cholestanone oxime, the 3-aminocholestanes, and the four new steroids were submitted to the

\* Received August 21, 1959, from the School of Pharmacy, University of Maryland, Baltimore 1.

This paper was awarded first prize for the Southern District in the 1959 Lunsford-Richardson Awards sponsored by Vick Chemical Co.

Abstracted from a thesis submitted by Robert E. Havranek to the Graduate Faculty of the University of Maryland, School of Pharmacy, in partial fulfillment of the requirements for the degree.

† Noxzer

Appreciation is expressed to the Research Institute for the analytical data. Presented to the Scientific Section, A. P. A., Cincinnati meeting, August 1959.

National Service Center for Cancer Chemotherapy for tumor screening. None showed activity.

### EXPERIMENTAL<sup>1</sup>

**3 $\beta$  - (2 - Hydroxyethylamino)cholestane.** — In a 2-L, three-necked flask, fitted with a stirrer, condenser, dropping funnel, and electric heating mantle, was placed 20 Gm (0.051 mole) of 3 $\beta$ -aminocholestane dissolved in 400 cc of purified benzene and 53 Gm (0.5 mole) of anhydrous sodium carbonate.

The mixture was heated to reflux with rapid stirring and 40 Gm (0.5 mole) of 2-chloroethanol added in four 10-Gm portions during the first three hours of the reaction period. Refluxing was continued for ten hours more, the mixture cooled, and the sodium carbonate-sodium chloride precipitate filtered. Benzene was removed, under reduced pressure, with a Borg revolving evaporator. The warm residue was sprayed with ether and a white precipitate of 3 $\beta$ -(2-hydroxyethylamino)cholestane formed. The precipitate was filtered. Later 12 Gm of unreacted 3 $\beta$ -aminocholestane was recovered from the ether solution.

The precipitate was dissolved in hot absolute ethanol and simultaneously treated with potassium carbonate and charcoal. The solution was filtered hot and the ethanol removed under reduced pressure. The residue was recrystallized from 4:1 acetone-ethanol. Cooling by refrigeration for a period of ten hours yielded 5.0 Gm (54.9%) of white well-formed needles (yield was calculated on the basis of the starting material not recovered), m p 172.5–173.5°,  $[\alpha]_D^{25} + 20^\circ \pm 0.1^\circ$  (1% in chloroform).

*Anal*—Calcd for  $C_{29}H_{53}NO$ : C, 80.68, H, 12.38, N, 3.25. Found: C, 80.64, H, 12.24, N, 3.17.

**3 $\alpha$  - (2 - Hydroxyethylamino)cholestane.** — 3 $\alpha$ -(2-Hydroxyethylamino)cholestane was prepared from 20 Gm (0.051 mole) of 3 $\alpha$ -aminocholestane and 7 Gm (0.056 mole) of 2-bromoethanol by a modification of the method used to prepare 3 $\beta$ -(2-hydroxyethylamino)cholestane. Absolute ethanol was used as the reaction solvent in place of benzene.

The purification procedure was the same as for the 3 $\beta$  epimer.

Recrystallization of the product from 4:1 acetone-ethanol yielded 9.0 Gm (42.8%) of white platelets, m p 113.6–114.4°,  $[\alpha]_D^{25} + 24.5^\circ \pm 0.1^\circ$  (1% in chloroform).

<sup>1</sup> The melting points are corrected.

*Anal*—Calcd for  $C_{29}H_{53}NO$ : C, 80.68, H, 12.38, N, 3.25. Found: C, 80.83, H, 12.66, N, 3.17.

**3 $\beta$  - (2 - Chloroethylamino)cholestane hydrochloride.** — 3 $\beta$ -(2-Hydroxyethylamino)cholestane (3.0 Gm, 0.0069 mole) and 100 cc of freshly distilled benzene were placed in a 400 cc, two-necked flask fitted with a condenser and heating mantle. The mixture was heated to dissolve the reagent and 3.0 Gm (0.02 mole) of thionyl chloride was added.

A precipitate immediately formed which was probably the benzene-insoluble amine hydrochloride salt. The mixture was refluxed one hour, cooled, and the excess thionyl chloride and benzene removed under reduced pressure in a Borg revolving vacuum evaporator.

The residue was dissolved in hot absolute ethanol and treated with 6 Gm of Nuchar to remove the deep orange color. Subsequent filtration of the hot solution produced a clear, colorless solution that deposited colorless, fine needles on cooling with refrigeration. Successive concentrations of the mother liquor yielded more product to make a total yield of 1.7 Gm (57%), m p. above 300°, decompn.

*Anal*—Calcd for  $C_{29}H_{53}Cl_2N$ : C, 71.57, H, 10.98, total Cl, 14.57. Found: C, 71.48, H, 10.74, total Cl, 14.30.

**3 $\alpha$  - (2 - Chloroethylamino)cholestane hydrochloride.** — 3 $\alpha$ -(2-Chloroethylamino)cholestane hydrochloride was prepared from 2.0 Gm (0.0046 mole) of 3 $\alpha$ -(2-hydroxyethylamino)cholestane and 2.0 Gm (0.013 mole) of thionyl chloride by the same procedure described for the synthesis of 3 $\beta$ -(2-chloroethylamino)cholestane hydrochloride. White needles were obtained in a total yield of 1.25 Gm (63%), m p above 300°, decompn.

*Anal*—Calcd for  $C_{29}H_{53}Cl_2N$ : C, 71.57, H, 10.98, total Cl, 14.57. Found: C, 71.54, H, 11.26, total Cl, 14.40.

### REFERENCES

- (1) Hazen, G. P., *Dissertation Abstr.*, 1950, 449.
- (2) British Empire Cancer Campaign, Annual Report, 1956.
- (3) Vavasour, G. R., Bolker, H. I., and McKay, A. F., *Can. J. Chem.*, 30, 993 (1952).
- (4) Gensler, W. J., and Sherman, G. M., *J. Org. Chem.*, 23, 1227 (1958).
- (5) Philips, F. S., First Symposium on Chemical Biological Correlations, National Academy of Sciences—National Research Council, Washington, D. C. 1951, p. 409.
- (6) Ishidate, M., Current Research in Cancer Chemotherapy, No. 5, 1956.
- (7) Personal communication.
- (8) Hawroth, R. D., and Dodgson, D. P., *J. Chem. Soc.*, 1952, 67.
- (9) Shoppee, C. W., Evans, D. E., Richards, H. C., and Summers, G. H., *Chem. & Ind.*, 1954, 1535.
- (10) Shoppee, C. W., Evans, D. E., Richards, H. C., and Summers, G. H., *J. Chem. Soc.*, 1956, 1649.
- (11) Personal communication.

## Notes

### A Note on the Inverse Isotope Dilution Analysis of Salicylic Acid\*

By C. E. BRECKINRIDGE, Jr., and J. E. CHRISTIAN

USING THE LABELED derivative method of inverse isotope dilution, a procedure has been developed for the analysis of salicylic acid in submilligram quantities. A spectrophotometric method was utilized for the determination of the weight of the derivative. The sensitivity of three different methods of determining the radioactivity of  $I^{131}$  samples was investigated to determine the best counting procedure. Using equal aliquots of an  $I^{131}$  solution, a 2.5 mg./cm.<sup>2</sup> window Geiger-Mueller counter showed a net activity of 1,385 counts per minute. A one inch NaI crystal scintillation counter registered a net of 31,806 while a liquid scintillation spectrometer<sup>1</sup> yielded a net rate of 88,550 counts per minute. Thus the liquid scintillation spectrometer was adopted for use in this work.

Radioactive iodine monochloride (the reagent) was prepared as described by Swartz and Christian (1) with the exception that 20 mc. of  $I^{131}$  in the form of Na $I^{131}$  in basic Na<sub>2</sub>SO<sub>3</sub> was used, and only one distillation to complete dryness was carried out. These changes were made to increase the concentration and the specific activity of the product.

Samples of salicylic acid to be analyzed were prepared by dissolving a known weight of salicylic acid in glacial acetic acid. Appropriate aliquots of this solution containing the quantity of salicylic acid to be analyzed were placed in 7 cm.  $\times$  1 cm. test tubes and 1 ml. of the labeled reagent containing 425 mg. ICl was added. Since the quantities analyzed were 10 mcg. and 1 mcg., this represented a definite excess of ICl. The solution in the test tubes was heated in a water bath for twenty minutes at 70 to 80° with intermittent agitation by shaking. A known weight (2 mg.) of carrier (nonradioactive 3,5-diiodosalicylic acid) was then added. Heat was applied for five minutes to insure complete solution and intimate mixing. The solution was cooled to 50° and the derivative precipitated by adding distilled water dropwise. The mixture was digested at 40 to 50° for five minutes, then cooled to room temperature. The test tubes were placed in a centrifuge for five minutes, after which the supernatant liquid was discarded. The precipitate was washed with distilled water twice, dried, and crystallized from glacial acetic acid. The centrifuge was employed after each step. The crystals were washed with distilled water, dried, and dissolved in approxi-

TABLE I.—RESULTS OF ANALYSES OF SALICYLIC ACID IN 10 MCG. AND 1 MCG. AMOUNTS

Salicylic Acid Analyzed, <i>a, b</i> mcg.	Final Sp. A of 3,5-diiodosalicylic acid, c. p. m./mg.	Wt. of Salicylic acid by analysis, <i>c</i> mcg.	Error, %
10	$6.777 \times 10^3$	9.1	9
10	$6.533 \times 10^3$	8.8	12
10	$7.921 \times 10^3$	10.7	7
10	$6.605 \times 10^3$	8.9	11
10	$6.533 \times 10^3$	8.8	12
10	$6.457 \times 10^3$	8.7	13
1	$6.088 \times 10^2$	0.81	19
1	$6.752 \times 10^2$	0.90	10
1	$6.592 \times 10^2$	0.88	12
1	$6.672 \times 10^2$	0.89	11
1	$5.769 \times 10^2$	0.77	23
1	$6.088 \times 10^2$	0.81	19

*a, b* In each analysis, 2 mg. of carrier (3,5-diiodosalicylic acid) was added and the initial specific activity of the derivative was  $5.323 \times 10^5$  counts per minute per mg.

*c* Calculated using the equation:

$$\text{wt.} = G_2 \frac{SpAf}{SpAi - SpAf} \times \frac{138.12}{389.94}$$

where:  $G_2$  = weight of carrier added,  $SpAf$  = specific activity of the carrier-diluted derivative,  $SpAi$  = specific activity of the derivative as calculated from the specific activity of the reagent, 138.12 = the molecular weight of salicylic acid, 389.94 = the molecular weight of 3,5-diiodosalicylic acid

mately 3 ml. of ethyl alcohol. Exactly 3 ml. of the resulting solution was pipetted into a quartz cuvet and the concentration of the solution was determined, using the Beckman DU spectrophotometer. A spectral-concentration curve for 3,5-diiodosalicylic acid was previously prepared at 328 mμ, the point of maximum absorbance. The samples were then transferred quantitatively to 20-ml. Wheaton vials using the phosphor solution for rinsing. Sufficient phosphor solution was added to give approximately 18 ml. and the activity was determined by liquid scintillation (Tri-Carb). The activity of the reagent was determined at this time using the same procedure. Toluene was used as the solvent, PPO (4 Gm. per liter) as the phosphor, and POPOP (0.1 Gm. per liter) as the wavelength shifter in the preparation of the phosphor solution. The results obtained at the 10 mcg. and 1 mcg. levels using this procedure appear in Table I. As little as 1-mcg. amounts of salicylic acid were determined with an average error of 15.7%.

#### REFERENCE

- (1) Swartz, H. A., and Christian, J. E., *THIS JOURNAL* 47, 702(1958).

\* Received August 21, 1959, from the Bionucleonics Department, Purdue University, Lafayette, Ind.

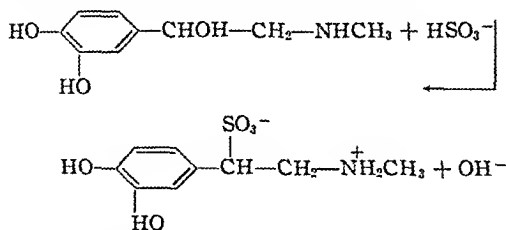
Presented to the Scientific Section, A. P. N. A., Cincinnati Meeting, August 1959.

<sup>1</sup> Packard Tri-Carb liquid scintillation spectrometer.

# A Note on the Preparation of 1-(3,4-Dihydroxyphenyl)-2-methylaminoethane Sulfonic Acid from Epinephrine\*

By LOUIS C. SCHROETER† and TAKERU HIGUCHI

KINETIC STUDIES (1, 2, 3) indicated that epinephrine reacts with sodium bisulfite or sulfite solution to yield a zwitter-ionic sulfonate:



The preparative value of this reaction and comprehensive physical constants of the product and its acetyl derivative have not been previously reported.

## EXPERIMENTAL

Epinephrine hydrochloride, 0.025 mole (5.492 Gm.) and sodium bisulfite, 0.025 mole (2.602 Gm.) were dissolved in about 50 ml. distilled water; the pH was adjusted to 6.8 by the addition of standard sodium hydroxide. The solution was introduced into a three-necked flask fitted with a dropping funnel, reflux condenser, and a tube for flushing the solution with nitrogen. The reaction mixture (ca. 80 ml.) was refluxed for six hours while keeping it flushed with nitrogen. Standard hydrochloric acid (1 *N*) was added periodically to maintain pH range 6.7 to 6.9. After refluxing, excess water (ca. 65 ml.) was distilled from the solution. The white crystalline precipitate which separated on cooling the solution was recrystallized from 1 *N* hydrochloric acid and twice from distilled water. Yield: 5.28 Gm. (85.5%).

*Anal.*—Calcd. for  $\text{C}_9\text{H}_{13}\text{NO}_5\text{S}$ : C, 43.71; H, 5.29; N, 5.66; O, 32.36; S, 12.97. Found: C, 43.72; H, 5.79; N, 5.50; O, 31.90; S, 13.05. The product melted with decomposition at 263° (Kofler hot stage) and exhibited the following optical crystallographic properties: system, orthorhombic; class, dipyramidal; refractive indexes,  $\alpha = 1.544$ ,  $\beta = 1.660$ ,  $\gamma = 1.696$ ; optic sign, negative;  $2V = 55^\circ$  (calculated from  $\alpha$ ,  $\beta$ , and  $\gamma$ ). X-ray powder diffraction data shown in Table I were obtained using copper radiation with a nickel filter (1.5405 Ångströms). The pKa determined in water was 8.95. Ultraviolet spectrum in water shows maxima at 228 and 278  $\mu$ ;  $\epsilon_{228} = 6,650$ ,  $\epsilon_{278} = 2,850$ .

1-(3,4-Diacetoxyphenyl)-2-methylaminoethane Sulfonic Acid.—Acetylation of 1-(3,4-dihydroxy-

phenyl)-2-methylaminoethane sulfonic acid was accomplished by suspending 2 mM (495 mg.) of the latter in 3.0 ml. glacial acetic acid. Two milliliters acetic anhydride and two drops concentrated hydrochloric acid were added to the solution and the latter refluxed for about twenty minutes. The white crystalline precipitate which separated on cooling the solution was recrystallized from glacial acetic acid. Yield: 413 mg. (62.5%).

TABLE I.—X-RAY POWDER DIFFRACTION DATA 1-(3,4-DIHYDROXYPHENYL)-2-METHYLAMINOETHANE SULFONIC ACID

d, Å	I/I <sub>1</sub>	d, Å	I/I <sub>1</sub>
8.34	0.43	3.64	0.32
7.40	0.48	3.52	0.26
6.18	0.90	3.40	1.00
5.58	0.85	3.33	0.34
5.29	0.87	3.21	0.46
4.69	0.35	3.09	0.29
4.58	0.33	2.81	0.58
4.32	0.60	2.65	0.39
4.17	0.41	2.44	0.34
4.01	0.82	2.24	0.31
3.75	0.83	..	..

TABLE II.—X-RAY POWDER DIFFRACTION DATA 1-(3,4-DIACETOXYPHENYL)-2-METHYLAMINOETHANE SULFONIC ACID

d, Å	I/I <sub>1</sub>	d, Å	I/I <sub>1</sub>
15.22	0.85	4.73	0.58
9.28	0.77	4.59	0.43
7.48	0.75	4.27	0.63
6.23	1.00	3.91	0.64
5.83	0.81	3.75	0.76
5.47	0.55	3.53	0.55
5.04	0.91	3.36	0.39
4.90	0.56	3.13	0.38
..	..	3.06	0.47

*Anal.*—Calcd. for  $\text{C}_{13}\text{H}_{17}\text{NO}_7\text{S}$ : C, 47.13; H, 5.13; N, 4.23; O, 33.80; S, 9.68. Found: C, 47.21; H, 5.16; N, 3.93; O, 33.67; S, 9.48. Acetyl determination: theory, 25.98%. Found, 26.74%. The derivative melted 212 to 213° (Kofler hot stage) and exhibited the following crystallographic properties: system, triclinic; refractive indexes,  $\alpha = 1.480$ ,  $\beta = 1.502$ ,  $\gamma = 1.562$ ; optic sign, positive;  $2V = 65^\circ$  (calculated from  $\alpha$ ,  $\beta$ , and  $\gamma$ ). X-ray powder diffraction data shown in Table II were obtained using copper radiation with a nickel filter (1.5405 Ångströms). The pKa determined in water was 8.30. Ultraviolet spectrum in water shows maxima at 263 and 269  $\mu$  with a shoulder at 216  $\mu$ ;  $\epsilon_{216} = 8,500$ ,  $\epsilon_{270} = 411$ .

## REFERENCES

- (1) Schroeter, L. C., Higuchi, T., and Schuler, E. E., *THIS JOURNAL*, 47, 723 (1958).
- (2) Higuchi, T., and Schroeter, L. C., *ibid.*, 48, 535 (1959).
- (3) Higuchi, T., and Schroeter, L. C., *J. Am. Chem. Soc.*, in press.

\* Received December 7, 1959, from the School of Pharmacy, University of Wisconsin, Madison and the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

† Research Associate, The Upjohn Co.  
The authors wish to thank Dr. John W. Shell, The Upjohn Co., for helpful crystallographic suggestions and Dr. William A. Struck, The Upjohn Co., and members of his staff, for analyses.

# A Note on the Biosynthesis of Clavine Alkaloids in *Claviceps purpurea* Strain 15B\*

By L. R. BRADY and V. E. TYLER, Jr.

The data obtained with a replacement culture technique indicate two types of precursors are required for the formation of the ergoline nucleus in ergot strain 15B. L-Tryptophan was shown to be the most efficient aromatic precursor. DL-Phenylalanine and DL- $\beta$ -phenyllactic acid were found to suppress alkaloid accumulation.

IT HAS BEEN ESTABLISHED that different clavine-producing strains of *Claviceps* exhibit different physiological responses (1). The two strains<sup>1</sup> which have been characterized in this laboratory show a marked contrast in their response to a reduced phosphate concentration in the growth medium and to additions of sucrose and DL-phenylalanine. The biosynthesis of the clavine alkaloids in strain 47A has been investigated using a replacement culture technique (2). It was shown that in this strain the ergoline nucleus is formed from two different types of precursors; an aromatic nucleus which may be derived from phenylalanine, tyrosine, or tryptophan, and a compound closely related to succinic acid or easily derived from it.

Since physiological studies had revealed that one of the amino acid precursors suppressed alkaloid accumulation in strain 15B, it was considered desirable to study the biosynthesis of the alkaloids in this organism by a replacement culture technique. This would separate a suppression resulting from gross physiological effects on growth from that associated more directly with the biosynthetic sequence which terminates with the clavine alkaloids.

additions to the replacement media and the average alkaloid concentrations of the original growth media and the replacement media are recorded in Table I. Four replicate cultures were employed for each addition.

Paper chromatographic examination of the media indicated agroclavine was the only indole alkaloid which was present in detectable quantities, the conclusion of the incubation period of the replacement media, tryptophan was detected in the cultures to which it had been added; a nonaromatic indole compound ( $R_f$  0.85) was also observed in the media when this amino acid was present in combination with succinic acid. A detectable trace of phenylalanine remained in the media to which it had been added together with the organic acid. Tyrosine and phenylalanine were not detected in other media to which they had been added.

## DISCUSSION

The data are consistent with the conclusion that two types of precursors are involved in the biosynthesis of the clavine alkaloids (2). However, in strain 15B DL-phenylalanine is not a satisfactory aromatic precursor, and on an equimolar basis L-tryptophan is more efficient than L-tyrosine. The responses to the additions of the amino acids contrast with those elicited in strain 47A in which phenylalanine, tyrosine, and tryptophan were served to be aromatic-type precursors of nearly equal efficiency.

The suppression of agroclavine accumulation by DL-phenylalanine and DL- $\beta$ -phenyllactic acid is of

TABLE I.—CONCENTRATION OF CLAVINE ALKALOIDS IN CULTURE MEDIA

Replacement Medium Mineral Solution plus	Growth Medium, $\gamma$ /ml., Agroclavine	Replacement Medium, $\gamma$ /ml., Agroclavine	Clavine Alkaloids, %, Estimated Factor	Corrected Content of Replacement Medium, $\gamma$ /ml., Agroclavine
DL-Phenylalanine	96.3	36.6	100	36.6
No additions	83.0	40.1	100	40.1
DL-Phenylalanine, succinic acid (1)	114.6	40.6	100	40.6
DL-Phenylalanine, succinic acid (2)	155.5	50.8	100	50.8
L-Tyrosine	87.1	56.4	100	56.4
DL- $\beta$ -Phenyllactic acid, succinic acid	126.0	61.8	100	61.8
Succinic acid	154.7	92.3	100	92.3
L-Tyrosine, succinic acid	166.6	106.0	100	106.0
L-Tryptophan	94.0	257.7	50	128.9
L-Tryptophan, succinic acid	159.7	274.2	50	137.1

## EXPERIMENTAL

The ergot strain, strain 15B, which was employed in this investigation was isolated by Tyler from a sclerotium which had developed on *Agropyron semicostatum* Nees (3). The experimental conditions and manipulations have been described previously (2). The identity of the

considerable interest. Abe, *et al.* (4), reported that phenylacetic acid caused a similar retardation in the accumulation of clavine alkaloids in replacement culture; the ergot strain employed by them had also been isolated from an *Agropyron* host. These observations suggest that phenylalanine and certain structurally related compounds act as clavine alkaloid antimetabolites in strain 15B.

## REFERENCES

- (1) Brady, L. R., and Tyler, V. E., Jr., Unpublished data.
- (2) Brady, L. R., and Tyler, V. E., Jr., *Planta Med.*, **7**, 225 (1959).
- (3) Tyler, V. E., Jr., *THIS JOURNAL*, **47**, 787 (1958).
- (4) Abe, M., Yamano, T., and Kusumoto, M., *J. Agr. Chem. Soc. Japan*, **27**, 617 (1953).

\* Received November 16, 1959, from the Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle 5.

Abstracted from a dissertation submitted to the Graduate School of the University of Washington by L. R. Brady in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

<sup>1</sup> Cultures of these two strains of *Claviceps* have been deposited with the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

# A Note on the Effect of Gibberellic Acid on *Azotobacter indicus*\*

By LEO GREENBERG and JOHN TIRPAK

ALTHOUGH gibberellins are widely known as growth stimulants in higher plants, no effect on microorganisms was demonstrated in studies by Brian, *et al.* (1), and Ciferri and Bertossi (2). Interest in the influence of gibberellins was aroused by the report of Lu, *et al.* (3), that numerous colonies of *Azotobacter* appeared on sodium albuminate agar plates upon cultivation of soil treated with ethanolic gibberellic acid. In view of the fact that, to the best of our knowledge, no laboratory study of *Azotobacter* and gibberellins has appeared in the literature, such an investigation was undertaken.

Purified gibberellic acid was obtained commercially (Sigma Chemical Co.) and *Azotobacter* species purchased from the American Type Culture Collection. Preliminary studies showed that only *Azotobacter indicus* (ATCC 9540) grew in a diffuse fashion throughout the test medium with a minimum of pellicle formation or discoloration by pigment formation or waste products.

Dilutions of gibberellic acid were made with sterile isotonic saline which was shown to have no detectable effect on the normal growth of the organism. *A. indicus* was maintained on nitrogen-free mannitol agar in Kille flasks and a standard liquid inoculum prepared by adding one loopful of surface growth to 25 ml. of sterile mannitol-tap water medium assigned a value of 100% transmittance at 420 m $\mu$  on a Coleman spectrophotometer. This standard inoculum was incubated at 25° until an absorbance of 80% transmittance was achieved, a process usually requiring about seventy-two hours.

Two sets of optically matched tubes containing 25 ml. of sterile mannitol-tap water medium and assigned the arbitrary value of 100% transmittance at 420 m $\mu$  of the spectrophotometer were used. Five milliliters of the appropriate dilution of gibberellic acid was added to one set of tubes and 5 ml. of sterile saline was added to the control set. All tubes then received 0.1 ml. of bacterial inoculum and kept well mixed at 25°. Changes in optical transmittance were recorded daily for ten days.

Repeated trials showed that in almost all cases, a distinct increase in growth as measured by decrease in optical transmittance could be demonstrated in the presence of gibberellic acid. A typical comparison of experimental to control series can be seen in

\* Received January 19, 1960, from the Brooklyn College of Pharmacy, Long Island University, Brooklyn, N. Y.

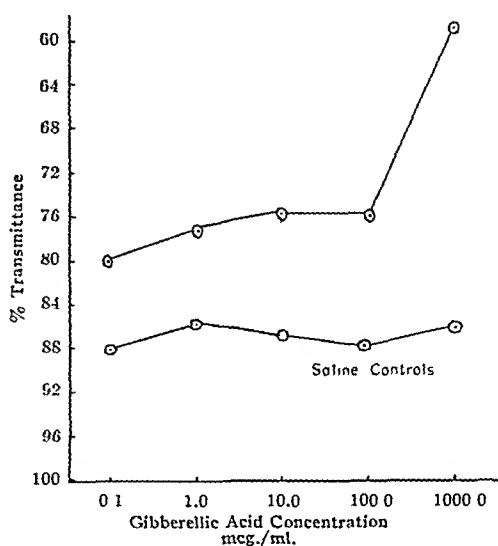


Fig. 1.—Effect of increasing concentrations of gibberellic acid on the growth of *Azotobacter indicus* compared to replicate saline controls (ten days incubation).

Fig. 1. To determine if this stimulatory effect could be demonstrated in other media, a quintuplicate run using Ashby's medium and gibberellic acid in a concentration of 1000 mcg./ml. was performed. After ten days' incubation with the test organism, experimental tubes gave a mean transmittance value of  $72.7 \pm 0.8\%$  compared to a control value of  $77.3 \pm 1.7\%$ . This difference is significant,  $P$  being less than 0.05.

On the basis of our determinations, it seems likely that solutions of gibberellic acid have a stimulating effect upon *Azotobacter indicus*, but the lack of linearity and the occasional variations in extent of stimulation indicate that this effect is a complex physiological one.

## REFERENCES

- (1) Brian, P. W., Elson, G., and Radley, M., *J. Sci. Food & Agr.* 33 114 (1957).
- (2) Ciferri, O., and Bertossi, F.
- (3) Lu, K. C., Gilmour, C. M., Zagallo, A. C., and Bollen, W. B., *Nature*, 181, 189 (1958).



## Book Notices

*Chemical Analysis* Vol 10 The Chemical Analysis of Air Pollutants By MORRIS B JACOBS Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N Y, 1960 xviii + 430 pp 15 X 23 cm Price \$13 50

This volume of a series of monographs on analytical chemistry and its applications presents detailed methods for the determination of the kind and amount of air contaminants. The presentation for each method follows the pattern 1 General methods for sampling with procedures for the determination of air and gas volume, quantity, and velocity 2 Analysis of the settled particulate matter and also the suspended particulate matter 3 Methods for the analysis of gaseous and vapor contaminants of the atmosphere, including radiochemical methods. Appended are tables of analytical data relating to suspended particulate matter in the air, sootfall, gaseous contaminants, and motor vehicle exhaust gas composition. A subject index is included.

*Soviet Research in Pharmaceutical Chemistry* Part I, Pharmaceutical Chemistry, Part II, Medicinal Chemistry, Part III, Pharmacognosy English translation Consultants Bureau, Inc., 227 West 17th St., New York 11, N Y, 1959 603 pp 21 X 27 cm Paperbound Price Part I, \$12, Part II, \$45, Part III, \$10, Complete collection \$60

These additions to the Chemistry Collection Series include translations of selected reports that have appeared in Soviet publications. The pharmaceutical chemistry part includes nine papers on ion exchange and its applications, particularly relating to the behavior of ion exchange resins with antibiotics. Fourteen additional miscellaneous reports are included. Part II includes thirteen papers on anti-infective compounds, eight papers on steroids and related compounds, thirty five papers on heterocyclic compounds, and twenty miscellaneous reports. Part III, entitled Pharmacognosy, includes seventeen papers on plant alkaloids, one on pectic substances of sunflower, and one on corioborin P.

*Ferment Hormone Vitamine* Band I Fermente 3rd ed By ROBERT AMMON and WILHELM DIRSCHERL Georg Thieme Verlag, Stuttgart, Germany, 1959 Agents in U S and Canada, Intercontinental Medical Book Co., 381 Fourth Ave., New York 16, N Y xx + 564 pp Price \$22 85 Subscription price \$19 45

This third edition, in three volumes, is the first revision since 1943. Volume 1, on ferments or enzymes, is, of necessity, a completely rewritten text. It attempts to relate significant relationships between biological catalysis and enzymes. This is developed with regard to individual and group enzyme activities. The book is intended for use by biochemists, medicinal chemists, and others in related chemical fields.

*Lymphocytes and Mast Cells* By MARGARET A KELSALL and EDWARD D CRABB The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1959 xvi + 399 pp 15 X 23 cm Price \$8

This book is a comprehensive treatise on two cells. Lymphocyte—a specialized cell which synthesizes stores, and transports nucleoprotein for maintenance, growth, or secretion by other cells, and Mast cell—chief significance is ability to synthesize, store and release histamine and heparin, but its production of mucopolysaccharide and its possible relation with serotonin is also discussed. A bibliography of 1,142 references cited in the text and a subject index are appended.

*Organic Reactions* Vol 10 Edited by ROGER ADAMS John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N Y, 1959 vii + 563 pp 15 X 23 cm Price \$12

This volume of "Organic Reactions," the tenth since the series was started in 1942, continues the comprehensive treatment of selected subjects. The ninth volume of the series was reviewed in THIS JOURNAL, 47, 155(1958). The present volume covers The coupling of diazonium salts with aliphatic carbon atoms, the Japp Klingemann reaction, and a very complete treatment of The Michael reaction. This latter chapter covers 379 pages and includes 1,045 references. The excellent style of presentation developed for this series is continued. A subject index for Volume 10 and cumulative author and chapter heading indexes are appended.

*The Preparation of Medical Literature* By LOUISE MONTGOMERY CROSS J B Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1959 xx + 451 pp 15 X 22.5 cm Price \$10

This book is designed to be used as a desk reference for details on practical techniques in the preparation of medical reports, articles, and books, in which physicians and allied scientists communicate clinical experience and scientific investigation to their colleagues. Writers for publication in any scientific field will find this book useful. Chemists should note that abbreviations for names of journals are from the "Quarterly Cumulative Index Medicus" and the "Current List of Medical Literature." As of January 1960, these two reference publications were replaced by "Index Medicus" (new series) published monthly by the National Library of Medicine, and annual cumulations of the index will be published by AMA. The journal abbreviations in the new "Index Medicus" follow, with some variations, those used in "Quarterly Cumulative Index Medicus." Writers of material for publication in the chemical fields should rely on the abbreviations recommended by *Chemical Abstracts*.

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49

JUNE 1960

NUMBER 6

## Investigation of Drug Release from Solids III\*

### Effect of a Changing Surface-Weight Ratio on the Dissolution Rate

By DALE E. WURSTER and JAMES A. SEITZ†

A dissolution study was conducted in which the surface area-weight ratio of a dissolving solid was not held constant. Cylindrical tablets containing large pores were employed to follow the dissolution process. Studies in distilled water indicated that the surface area of the tablet pore was incompletely exposed to the solvent due to occlusion by air. Solutions with a lower surface tension than water were capable of wetting the entire surface area and a corresponding increase in the dissolution rate was obtained. When the air was evacuated from the artificially produced tablet pores the solvent was permitted to come in contact with the entire surface and the dissolution rate increased. Dissolution from the pore surface occurred at a slower rate than from the exterior surface due to the longer diffusional pathway of the solute molecules.

**T**HE FACTORS influencing the dissolution rate of a slightly-soluble medicinal agent are many and varied but possibly none of them play a more important role than the surface characteristics (1-3) of the solid particle.

The dissolution behavior of materials obtained from granulation, lyophilization, and other procedures would be especially difficult to predict due to the variation in fissures and pores introduced into the solids during the drying stages. Since the dissolution rate is proportional to the surface area of the dissolving particle, measurements of the total surface area as well as the area calculated from the gross particle size might lead to erroneous calculations of the rate in a diffusion-controlled process.

The present investigation was, therefore, undertaken to study the factors influencing dissolution of slightly-soluble solids in which the surfaces are of a varying degree of availability to the solvent.

#### METHOD OF STUDY

Previous workers (4-6) have generally circumvented the problem of a changing surface either by maintaining it relatively constant or by using geometric shapes where the surface could be readily calculated during the dissolution process. Equations describing dissolution kinetics show that the dissolution rate is directly proportional to the area exposed to the solvent under conditions of uniform agitation and a constant shape. Therefore, particles with a highly irregular or fissured surface should have a faster overall rate of solution, if the entire surface is exposed to the solvent, than spherical particles where the surface area per unit weight is minimal.

In order to study the availability of the surface to the solvent, model porous particles were prepared by introducing artificial pores into cylindrical compressed tablets. The initiating work of Parrott (5) indicated that the porosity of an isotropic crystalline mass such as that in compressed tablets was of sufficiently low magnitude that the surface-weight ratio remained constant and the tablet could be treated as a homogeneous solid. However, in the case where the pores are sufficiently large or the conditions such that the pore surface participates in the dissolution process, a varying surface-weight ratio results. The availability of the pore surface was studied by following the dissolution rate of cylindrical benzoic acid tablets in distilled water, in a sodium lauryl sulfate solution, and gas-evacuated tablets in distilled water.

\* Received April 25, 1958, from the University of Wisconsin, School of Pharmacy, Madison 6.

This paper is based on a dissertation submitted by James A. Seitz to the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† University of Wisconsin fellow.

Presented to the Scientific Section, A Pitt A Los Angeles meeting, April 1958.

The mathematical interpretation is based on the Hixson-Crowell (7, 8) equation

$$dw/dt = -3KS = -3Ka W^{2/3} \quad (\text{Eq. 1})$$

which upon integration yields

$$Kat = W_0^{1/3} - W^{1/3} \quad (\text{Eq. 2})$$

where  $K$  is the velocity constant,  $S$  is surface area,  $a$  is the surface-weight constant,  $t$  is time, and  $W_0$  and  $W$  are the weights at  $t = 0$  and  $t$ , respectively. The constant,  $a$ , for a cylinder or sphere, where the shape remains constant throughout the dissolution study, can be determined mathematically or graphically as  $S = a W^{2/3}$ . When the surface-weight ratio is not constant, as in the case of solids with pores large enough to be involved in the dissolution process, it is possible to use a reduced form of Eq. 2,

$$K't = W_0^{1/3} - W^{1/3} \quad (\text{Eq. 3})$$

where  $K'$  is comparable to the constants  $Ka$ . Experimental evidence indicates  $K'$  to be constant during the initial phase of the dissolution process. The velocity constant  $K$  during this time period can be evaluated by determining the surface-weight ratio at the same time.

## EXPERIMENTAL

**Procedure.**—The experimental procedure of Parrott (5) was followed except for minor alterations. During the dissolution procedure the tablet was removed from the solution at regular time intervals, rapidly dried, and the dimensions measured with an optic micrometer (Gaertner Scientific Corp.) on a rotating substage. The depth of the artificial pores was also determined. The diameter at the base of the pore was calculated by assuming the change in the depth of the pore was equal to the change in the diameter at the base. The dimensions were measured to 0.0001 cm., with the number of readings varying from 24 for the solid tablets to 84 for the 6-pore tablets. After weighing, the tablet was returned to a fresh 2,000-ml. quantity of solution for another cycle.

The 0.2% sodium lauryl sulfate solutions were prepared with freshly-boiled distilled water and then neutralized to phenol red indicator with 0.02 *N* HCl.

The gas-evacuated tablets were evacuated for not less than two hours with a Cenco-Pressovac vacuum pump. The tablet evacuation chamber was a modified  $19/32$  ground-glass joint with ground-glass stopcocks on either end. One stopcock opened to the vacuum pump and the other to the distilled water flask. A flask containing freshly-boiled distilled water was attached to the chamber while the water was still vaporizing to insure that no air would be absorbed on cooling. After the water cooled to the desired temperature it was allowed to envelop the tablet, and the contents of the chamber were immediately transferred to the agitated liquid in the dissolution vessel.

The tablets were compressed with an instrumented Stokes (model A-3) single punch tablet machine equipped with  $3/8$  inch flat punches. The tablets were all compressed with the same compressional force as recorded by oscillograph

recordings. The artificial pores were introduced by drilling holes in the flat surfaces of the tablets with a drill press using a 0.04-inch diameter drill. These holes of uniform depth were placed equidistant between the center and the edge of the tablet.

Using the procedures outlined above, the dissolution behavior of solid cylindrical benzoic acid tablets and tablets containing 1 to 6 large pores was investigated. The rate of solution of these tablets was studied in distilled water and 0.2% sodium lauryl sulfate solution. The dissolution rate of air-evacuated tablets in distilled water is also reported.

**Reagents.**—Benzoic acid, reagent grade; sodium lauryl sulfate U. S. P.

## RESULTS AND DISCUSSION

**Dissolution Study in Distilled Water.**—In the derivation of Eq. 3 it was assumed  $K'$  would be constant for the initial phase of the dissolution process. When  $W_0^{1/3} - W^{1/3}$  was plotted against time a straight line relationship was obtained which indicated this assumption to be correct. However, the same linearity would be obtained if the pores were occluded by air or other adsorbed gases. Likewise, the surface-weight ratio,  $a$ , and the velocity constant,  $K$ , would then be constant as observed for solid cylindrical tablets. Although the  $Ka$  values were generally increasing as more pores were introduced into the tablets, this increase was due largely to the surface-weight factor,  $a$ .

In evaluating the constant,  $a$ , an indication of the degree of participation of the internal pore surface was obtained when the experimental line was compared with a theoretical line, where it was assumed that the pore surface did not participate in the dissolution process. It can be seen in Fig. 1 that there was practically no difference between the experimental and the theoretical lines.

As shown in Table I, the rate of dissolution ( $3KS$ ) of the pore-containing tablets increased, but at a much slower rate than might be expected. With the introduction of six pores the surface area was increased over 20%, but the increase in the  $3KS$  value was less than 4%. Since the external surface accounted for about 96% of the benzoic acid dissolved, this suggests that the pores were largely occluded by air and the area was unavailable to the solvent. Pore diameter measurements supported this for only the orifices of the pores exhibited any enlargement during the dissolution study.

**Dissolution Studies in 0.2% Sodium Lauryl Sulfate Solution.**—To study the effect of a lowered surface tension on pore penetration and the dissolution rate, a 0.2% aqueous sodium lauryl sulfate solution was used. As in the case of distilled water the  $Ka$  value increased as more pores were introduced into the tablet, but unlike the previous study the surface-weight factor,  $a$ , was lower. In Fig. 2 the participation of the pores in the dissolution process can readily be seen when the experimental line is again compared with the previously mentioned theoretical line. During the dissolution process the pores became considerably enlarged. The dissolution from the cylindrical pores was not uniform but varied with the distance from the pore orifice, and at the completion of the dissolution study the shape of the pores was conical.

This alteration in the shape was theoretically expected due to the longer diffusional pathway of the solute molecules and the decreased agitation of the solvent in the pores which results in a progressively slower dissolution rate as the depth into the pore increases. It is obvious that the evaluation of  $K$  for porous solutes is related to the rate of change of the surface-weight factor,  $a$ . If the  $a$  value decreases and at the same time the  $Ka$  value increases, the velocity constant,  $K$ , will be greater and an increase in the rate of dissolution (3  $K$ S) (Table II) results.

**Dissolution Study of Gas-Evacuated Tablets in Distilled Water.**—When the tablets were gas evacuated the solvent was able to penetrate the pores and wet the entire surface. The result of gas evacuation was comparable to that observed by lowering the surface tension of the solvent (Fig. 2 and Table III). Visually, it was observed that the pores enlarged conically in a manner analogous to that with the sodium lauryl sulfate solution which, again, can be explained on the basis of the decreased

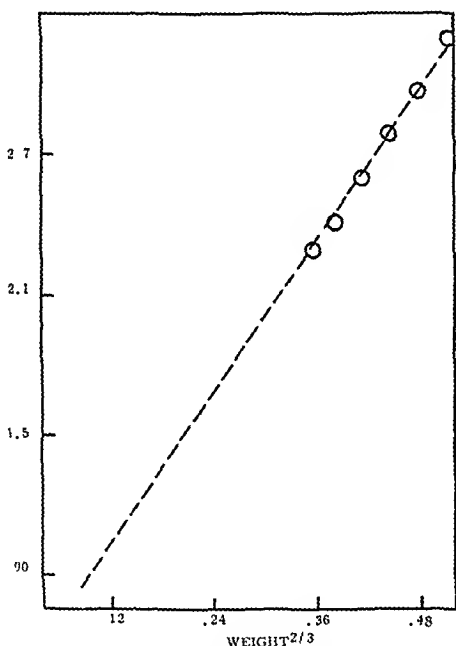


Fig. 1.—Comparison of theoretical with experimental surface-weight factor values for dissolution distilled water. --- Theoretical line based on the external surface of a 6-pore tablet participating in the dissolution process; O experimental data for a 6-pore tablet.

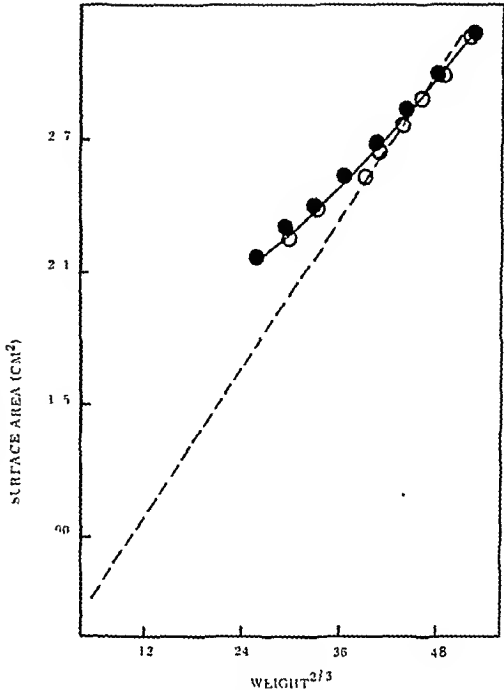


Fig. 2.—Comparison of theoretical with experimental surface-weight factor values. --- Theoretical line based on only the external surface of a 6 pore tablet participating in the dissolution process; — experimental line; O 6-pore tablet in 0.2% sodium lauryl sulfate solution; ● 6-pore air-evacuated tablet in distilled water.

dissolution rate in the pores resulting from the increased diffusional pathway of the solute molecules.

In the graphic determination of the  $Ka$  values ( $W_0^{1/3} - W^{1/3}$  vs. time) for the pore-containing gas-evacuated tablets a very slight departure from linearity occurred after one hour of dissolution. However, in all other cases, a straight line relationship was maintained throughout the entire required dissolution period one and one-half to two hours.

In all cases, with the introduction of pores into tablets the  $Ka$  values increased over those obtained for solid tablets but the increase varied with the experimental conditions. In sodium lauryl sulfate solutions the increase in the  $Ka$  values was the greatest, and in distilled water it was the least (Fig. 3). The difference in the  $Ka$  values for gas-

TABLE I.—COMPARISON OF DISSOLUTION RATES OF TABLETS WITH VARYING SURFACE AREAS IN DISTILLED WATER

Tablets	$Ka$	$a$	$K$	Total Surface Area, $S$ , $\text{cm}^2$	Surface Area of Pores $\text{cm}^2$	3 $K$ S, $\text{Gm}/\text{cm}^2/\text{hr.}$
Solid	0.0903	4.53	0.0199	2.491		0.1487
1 Pore	0.0987	4.76	0.0188	2.634	0.075	0.1486
2 Pores	0.0924	4.85	0.0191	2.693	0.186	0.1543
3 Pores	0.0924	5.21	0.0177	2.754	0.276	0.1462
4 Pores	0.0920	5.18	0.0178	2.849	0.371	0.1521
5 Pores	0.0960	5.68	0.0169	2.941	0.469	0.1491
6 Pores	0.0951	5.57	0.0171	3.004	0.564	0.1541

TABLE II—COMPARISON OF DISSOLUTION RATES OF TABLETS WITH VARYING SURFACE AREAS IN 0.2% SODIUM LAURYL SULFATE SOLUTION

Tablets	Ka	a	K	Total Surface Area, S, cm <sup>2</sup>	Surface Area of Pores, cm <sup>2</sup>	3 K.S, Gm/cm <sup>2</sup> /hr
Solid	0 0961	4 57	0 0210	2 552		0 1608
1 Pore	0 1039	4 63	0 0224	2 647	0 101	0 1779
2 Pores	0 1090	4 28	0 0255	2 697	0 189	0 2063
3 Pores	0 1118	4 19	0 0267	2 798	0 291	0 2241
4 Pores	0 1158	4 43	0 0262	2 869	0 373	0 2255
5 Pores	0 1187	4 15	0 0286	2 944	0 460	0 2526
6 Pores	0 1207	4 44	0 0272	3 022	0 530	0 2466

TABLE III—COMPARISON OF DISSOLUTION RATES OF AIR EVACUATED TABLETS WITH VARYING SURFACE AREAS IN DISTILLED WATER

Tablets	Ka	a	K	Total Surface Area, S, cm <sup>2</sup>	Surface Area of Pores, cm <sup>2</sup>	3 K.S, Gm/cm <sup>2</sup> /hr
Solid <sup>a</sup>	0 0903	4 53	0 0199	2 491		0 1487
2 Pores	0 0986	4 50	0 0219	2 671	0 183	0 1754
4 Pores	0 1043	4 60	0 0226	2 870	0 388	0 1946
6 Pores	0 1121	4 05	0 0277	3 008	0 563	0 2500

<sup>a</sup> Solid tablets were not air evacuated

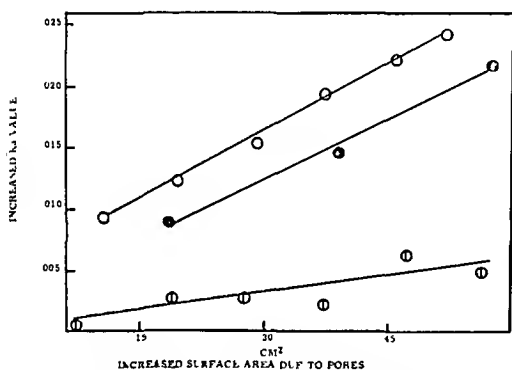


Fig. 3.—Effect of increased "effective" surface area on the Ka values: ○ 0.2% sodium lauryl sulfate solution; ● air-evacuated tablets in distilled water; ⊙ distilled water

evacuated tablets and tablets dissolved in 0.2% sodium lauryl sulfate solution can be attributed to the greater solubility of benzoic acid in the sodium lauryl sulfate solution. This increased solubility was substantiated by separate solubility tests.

A subsequent paper will deal with the dissolution behavior of lyophilized solids.

### SUMMARY

The dissolution rate of benzoic acid tablets with varying surface areas was investigated in distilled water and 0.2% sodium lauryl sulfate solution. The dissolution process of air-evacu-

ated tablets in distilled water was also investigated.

In distilled water the dissolution study indicated that pores in the benzoic acid tablets were somewhat occluded by air and, therefore, the surface area was not entirely available to the solvent.

The entire surface area of the pore-containing benzoic acid tablets participated in the dissolution process when 0.2% sodium lauryl sulfate was used. The increased dissolution rate was probably due to the lower surface tension, allowing penetration of the pores by the solvent.

Air evacuation of the benzoic acid tablet permitted the water to enter the pores. Results obtained were analogous to those obtained with 0.2% sodium lauryl sulfate solution.

The availability of the internal surface of porous particles to the solvent action is important in evaluating the effect of an increased surface area on the dissolution rate.

### REFERENCES

- (1) Dandon, M. L., and Mack, E., Jr., *J. Am. Chem. Soc.* 45, 2479 (1923).
- (2) Schurr, J., *J. chim. phys.* 2, 245 (1904).
- (3) Higuchi, W. I., Parrott, E. L., Wurster, D. E., and Higuchi, T., *This Journal*, 47, 376 (1958).
- (4) Nelson, E., *ibid.* 46, 607 (1957).
- (5) Parrott, E. L., Wurster, D. E., and Higuchi, T., *ibid.* 44, 269 (1954).
- (6) Edwards, L. J., *Trans. Faraday Soc.* 47, 1191 (1951).
- (7) Hixson, A. W., and Crowell, J. H., *Ind. Eng. Chem.* 23, 923 (1931).
- (8) *Ibid.* 23, 1002 (1931).

# The Effect of Surfactants on Drug Stability I\*

By S. RIEGELMAN

The effect of surfactants on the rate of hydrolysis of esters, using benzocaine as an example, is reported for a homologous series of alkali-stable nonionic surfactants, and with cationic and anionic surfactants. It was found that the hydrolysis rate of the ester in the alkali-stable nonionic surfactants varies with the concentration of the surfactant more than with the length of the polyoxyethylene chain of the surfactant. Hydrolysis apparently takes place within the micelle, as well as in the aqueous phase. Anionic and cationic surfactants appear to stabilize the drug to base catalysis, resulting in an eighteen-fold increase in half-life in 5 per cent lauryl sulfate solutions. A dilute solution of the quaternary, cetyl trimethyl ammonium bromide, slightly accelerates the rate of hydrolysis.

THE MICELLULAR AGGREGATES of surfactants possess the well-known property of increasing the solubility of water-insoluble organic substances. Many studies have been made, particularly with the nonionic group of surfactants, as to their usefulness as solubilizing agents for antibacterial agents (1-5), oil-soluble vitamins (6-8), steroids (9), estrogens (10), and for other substances. Several studies have been undertaken to clarify the effect solubilization has on drug availability. These include the release of phenols (2, 3), iodine (11), parabens (4, 5), and estrogens (12). Little is known, however, of the effect solubilization will have on the stability of a drug to hydrolytic attack. The solubilized drug may be held deep within the hydrocarbon interior of the micelle. Under these conditions, it is probable that the hydroxyl or hydrogen ions will not be able to make contact with the solubilized drug, and no hydrolytic cleavage will result. Solubilization may take place by other mechanisms, i. e., surface adsorption and short or deep penetration of the palisade layers of the micelle. Under the latter conditions, acid or base catalysis may or may not be able to take place. If the surface of the micelle is highly hydrated, nonionic surfactants may allow the catalytic acidic or basic ion to penetrate and to make contact with the solubilized drug within the polyoxyethylene chains. The length of the polyoxyethylene chain, the concentration of the surfactant and of the drug should all influence the rate of hydrolysis under standard conditions. Micelles of charged surfactants may have a different effect on the hydrolysis rate, since coulombic forces now play a predominant role in determining whether the catalytic ion may be able to invade the micelle to reach the solubilized

drug. In all of the cases, the amount of drug in the free aqueous phase will undergo base or acid catalysis and in many cases may be the only site of hydrolysis.

Benzocaine was selected as the ester chosen for study in this preliminary report. Higuchi and Lachman (13) carried out a study of the base catalyzed hydrolysis of benzocaine in water in a complex with caffeine. The presence of 2.5 per cent of caffeine in the system resulted in reduction of the hydrolytic rate to less than one-fifth of that in the absence of the caffeine. Their results indicated that the complexed form of the drug does not undergo any perceptible cleavage of the ester linkage.

The present study will show that the use of solubilization surfactants to solubilize a major portion of a drug can result in as much as an eighteen-fold stabilization of the drug to hydrolysis. Evidence is gathered which indicates that the drug undergoes base catalysis even within the nonionic surfactant micelle.

## EXPERIMENTAL

**Reagents.**—*Benzocaine*.—U. S. P. product, recrystallized from ethyl alcohol and water, m. p. 92-93°.

**Sodium Lauryl Sulfate.**—A U. S. P. grade (Duponol C, DuPont de Nemours) was recrystallized from hot absolute ethyl alcohol and washed with ether. The compound was substantially free from extraneous electrolyte and free lauryl alcohol as determined by surface tension studies. It had a melting point of 175-176° (14). A portion of the sample was converted into the free acid by passage through an acid-activated cation exchange resin (Dowex 50) until the filtrate reached a pH of 7. The neutralization equivalent was found to be 292.7; theoretical, 288.4.

**Cetyl Trimethyl Ammonium Bromide.**—A commercial sample (J. T. Baker Chem. Co.) was twice recrystallized from hot water, m. p. 237-238° (decompn.).

**Cetyl Alcohol Polyoxyethylene Ethers.**—Commercial samples of the compounds of ethylene oxide content ranging from 10 to 60 moles of ethylene oxide per mole of cetyl alcohol were selected for

\* Received August 21, 1959, from the School of Pharmacy, University of California Medical Center, San Francisco 22, Calif.

The author wishes to express his appreciation for the assistance given by Mr. Richard Penna and Mr. John Kobayashi in certain portions of this experiment.

Presented to the Scientific Section, A. P. A., Cincinnati meeting, August 1959.

TABLE II.—COMPARISON OF DISSOLUTION RATES OF TABLETS WITH VARYING SURFACE AREAS IN 0.2% SODIUM LAURYL SULFATE SOLUTION

Tablets	Ka	a	K	Total Surface Area, S, cm <sup>2</sup>	Surface Area of Pores, cm <sup>2</sup>	3 KS, Gm/cm <sup>2</sup> /hr
Solid	0 0961	4 57	0 0210	2 552		0 1608
1 Pore	0 1039	4 63	0 0224	2 647	0 101	0 1779
2 Pores	0 1090	4 28	0 0255	2 697	0 189	0 2063
3 Pores	0 1118	4 19	0 0267	2 798	0 291	0 2241
4 Pores	0 1158	4 43	0 0262	2 869	0 373	0 2255
5 Pores	0 1187	4 15	0 0286	2 944	0 460	0 2526
6 Pores	0 1207	4 44	0 0272	3 022	0 530	0 2466

TABLE III —COMPARISON OF DISSOLUTION RATES OF AIR EVACUATED TABLETS WITH VARYING SURFACE AREAS IN DISTILLED WATER

Tablets	Ka	a	K	Total Surface Area, S, cm <sup>2</sup>	Surface Area of Pores, cm <sup>2</sup>	3 KS, Gm/cm <sup>2</sup> /hr
Solid <sup>a</sup>	0 0903	4 53	0 0199	2 491		0 1487
2 Pores	0 0986	4 50	0 0219	2 671	0 183	0 1754
4 Pores	0 1043	4 60	0 0226	2 870	0 388	0 1946
6 Pores	0 1121	4 05	0 0277	3 008	0 563	0 2500

<sup>a</sup> Solid tablets were not air evacuated

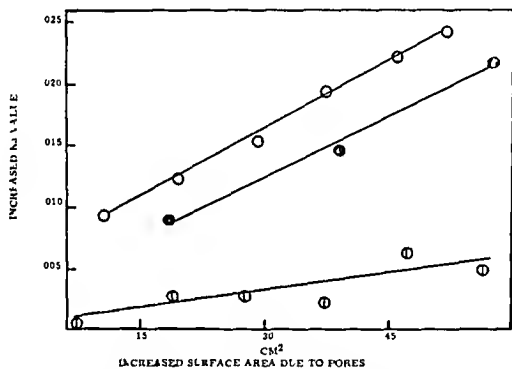


Fig 3.—Effect of increased "effective" surface area on the Ka values ○ 0.2% sodium lauryl sulfate solution; ● air-evacuated tablets in distilled water; ⊙ distilled water

evacuated tablets and tablets dissolved in 0.2% sodium lauryl sulfate solution can be attributed to the greater solubility of benzoic acid in the sodium lauryl sulfate solution. This increased solubility was substantiated by separate solubility tests.

A subsequent paper will deal with the dissolution behavior of lyophilized solids.

SUMMARY

The dissolution rate of benzoic acid tablets with varying surface areas was investigated in distilled water and 0.2% sodium lauryl sulfate solution. The dissolution process of air-evacu-

ated tablets in distilled water was also investigated.

In distilled water the dissolution study indicated that pores in the benzoic acid tablets were somewhat occluded by air and, therefore, this surface area was not entirely available to the solvent.

The entire surface area of the pore-containing benzoic acid tablets participated in the dissolution process when 0.2% sodium lauryl sulfate was used. The increased dissolution rate was probably due to the lower surface tension, allowing penetration of the pores by the solvent.

Air evacuation of the benzoic acid tablets permitted the water to enter the pores. Results obtained were analogous to those obtained with 0.2% sodium lauryl sulfate solution.

The availability of the internal surface of porous particles to the solvent action is important in evaluating the effect of an increased surface area on the dissolution rate.

REFERENCES

(1) Dundon, M. L., and Mack, E., Jr., *J. Am. Chem. Soc.* 45, 2479 (1923).

(2) Schurr, J., *J. chim. phys.*, 2, 245 (1904).

(3) Higuchi, W. J., Parrott, E. L., Wurster, D. E., and Higuchi, T., *This Journal*, 47, 376 (1958).

(4) Nelson, E., *ibid.*, 46, 607 (1957).

(5) Parrott, E. L., Wurster, D. E., and Higuchi, T., *ibid.*, 44, 269 (1954).

(6) Edwards, L. J., *Trans. Faraday Soc.*, 47, 1191 (1951).

(7) Hixson, A. W., and Crowell, J. H., *Ind. Eng. Chem.*, 23, 923 (1931).

(8) *Ibid.*, 23, 1002 (1931).

# The Effect of Surfactants on Drug Stability I\*

By S. RIEGELMAN

The effect of surfactants on the rate of hydrolysis of esters, using benzocaine as an example, is reported for a homologous series of alkali-stable nonionic surfactants, and with cationic and anionic surfactants. It was found that the hydrolysis rate of the ester in the alkali-stable nonionic surfactants varies with the concentration of the surfactant more than with the length of the polyoxyethylene chain of the surfactant. Hydrolysis apparently takes place within the micelle, as well as in the aqueous phase. Anionic and cationic surfactants appear to stabilize the drug to base catalysis, resulting in an eighteen-fold increase in half-life in 5 per cent lauryl sulfate solutions. A dilute solution of the quaternary, cetyl trimethyl ammonium bromide, slightly accelerates the rate of hydrolysis.

THE MICELLULAR AGGREGATES of surfactants possess the well-known property of increasing the solubility of water-insoluble organic substances. Many studies have been made, particularly with the nonionic group of surfactants, as to their usefulness as solubilizing agents for antibacterial agents (1-5), oil-soluble vitamins (6-8), steroids (9), estrogens (10), and for other substances. Several studies have been undertaken to clarify the effect solubilization has on drug availability. These include the release of phenols (2, 3), iodine (11), parabens (4, 5), and estrogens (12). Little is known, however, of the effect solubilization will have on the stability of a drug to hydrolytic attack. The solubilized drug may be held deep within the hydrocarbon interior of the micelle. Under these conditions, it is probable that the hydroxyl or hydrogen ions will not be able to make contact with the solubilized drug, and no hydrolytic cleavage will result. Solubilization may take place by other mechanisms, i. e., surface adsorption and short or deep penetration of the palisade layers of the micelle. Under the latter conditions, acid or base catalysis may or may not be able to take place. If the surface of the micelle is highly hydrated, nonionic surfactants may allow the catalytic acidic or basic ion to penetrate and to make contact with the solubilized drug within the polyoxyethylene chains. The length of the polyoxyethylene chain, the concentration of the surfactant and of the drug should all influence the rate of hydrolysis under standard conditions. Micelles of charged surfactants may have a different effect on the hydrolysis rate, since coulombic forces now play a predominant role in determining whether the catalytic ion may be able to invade the micelle to reach the solubilized

drug. In all of the cases, the amount of drug in the free aqueous phase will undergo base or acid catalysis and in many cases may be the only site of hydrolysis.

Benzocaine was selected as the ester chosen for study in this preliminary report. Higuchi and Lachman (13) carried out a study of the base catalyzed hydrolysis of benzocaine in water in a complex with caffeine. The presence of 2.5 per cent of caffeine in the system resulted in reduction of the hydrolytic rate to less than one-fifth of that in the absence of the caffeine. Their results indicated that the complexed form of the drug does not undergo any perceptible cleavage of the ester linkage.

The present study will show that the use of solubilization surfactants to solubilize a major portion of a drug can result in as much as an eighteen-fold stabilization of the drug to hydrolysis. Evidence is gathered which indicates that the drug undergoes base catalysis even within the nonionic surfactant micelle.

## EXPERIMENTAL

**Reagents.**—*Benzocaine*—U. S. P. product, recrystallized from ethyl alcohol and water, m. p. 92-93°.

*Sodium Lauryl Sulfate*—A U. S. P. grade (Dupanol C, DuPont de Nemours) was recrystallized from hot absolute ethyl alcohol and washed with ether. The compound was substantially free from extraneous electrolyte and free lauryl alcohol as determined by surface tension studies. It had a melting point of 175-176° (14). A portion of the sample was converted into the free acid by passage through an acid-activated cation exchange resin (Dowex 50) until the filtrate reached a pH of 7. The neutralization equivalent was found to be 292.7; theoretical, 238.4.

*Cetyl Trimethyl Ammonium Bromide*.—A commercial sample (J. T. Baker Chem. Co.) was twice recrystallized from hot water, m. p. 237-238° (decompn.).

*Cetyl Alcohol Polyoxyethylene Ethers*.—Commercial samples of the compounds of ethylene oxide content ranging from 10 to 60 moles of ethylene oxide per mole of cetyl alcohol were selected for

\* Received August 21, 1959, from the School of Pharmacy, University of California Medical Center, San Francisco 22, Calif.

The author wishes to express his appreciation for the assistance given by Mr. Richard Penna and Mr. John Kobayashi in certain portions of this experiment.

Presented to the Scientific Section, A. P. A., Cincinnati meeting, August 1959.



the study.<sup>1</sup> For convenience, the compounds will be designated as C-10, C-14, C-30, and C-60 to denote the average number of moles of ethylene oxide per molecule. All samples were dried at 60° under high vacuum. The C-14 sample was found to contain an impurity which absorbed in the ultraviolet region. This was reduced substantially by fractional crystallization of the compound from absolute diethyl ether, followed by complete removal of any ether held by the compound at 60° with high vacuum. This treatment unfortunately changed the average number of moles of ethylene oxide per molecule of surfactant, since the molecules with the longer polyoxyethylene chain preferentially precipitate.

**Apparatus.**—Beckman DU spectrophotometer with a constant temperature jacketed-cell chamber held at 30° ± 0.1°, and a model 11 Cary ultraviolet recording spectrophotometer.

**Analytical Method.**—The method employed in the present study consisted essentially of making a series of ultraviolet absorption measurements on a benzocaine solution undergoing hydrolysis at constant temperature and hydroxyl ion conditions. Although the ultraviolet absorption spectrum of benzocaine and its chromophore-containing hydrolytic product, *p*-aminobenzoic acid, are practically identical in neutral conditions, the salt form of *p*-aminobenzoic acid has a different electronic configuration so that its absorption spectrum is sufficiently different to permit quantitative analysis of mixtures of the salt and ester. The per cent of the residual ester was determined by analysis at the point of maximum absorbance of the benzocaine moiety (which shifted in each surfactant system) and at the isosbestic point between benzocaine and *p*-aminobenzoate in 0.04 *N* sodium hydroxide (see Fig. 1). The maxima of the solubilized benzocaine varied from 285 to 296 mμ. The isosbestic point varied from 271 to 277 mμ. The latter were time independent for each system studied as long as secondary oxidative reactions were absent. The solutions were read directly in the silica cuvet without dilutions.

**Experimental Procedure.**—Stock solutions of the drug and the surfactants were used to prepare 1, 10, and 33 mg. % solutions of benzocaine in the surfactant solutions. The surfactants were studied in concentrations ranging from 0.067 to 10%. The concentration of base was held at 0.04 *N*. Prior to the hydrolysis study, solutions containing the drug and surfactant in the absence of the base were examined for their ultraviolet absorption characteristics, using the Cary model 11 recording spectrophotometer. An identical sample completely hydrolyzed to *p*-aminobenzoate was also studied simultaneously. In this manner the isosbestic point and the absorption maximum were determined explicitly for each system studied.

Each hydrolytic experiment was carried out in the following manner. A volume of stock solution containing an amount of benzocaine equivalent to 1 mg. per 100 ml. of final solution was mixed with an amount of surfactant solution to yield the desired final concentration. These solutions were diluted with water to 14.4 ml. and equilibrated to a temper-

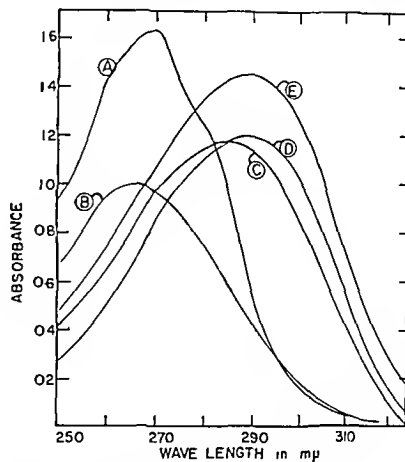


Fig. 1.—Ultraviolet absorption curves: A, benzocaine in hexane; B, *p*-aminobenzoate ion in 0.04 NaOH; C, benzocaine in water; D, benzocaine 0.53% surfactant C-14; E, benzocaine in 20% polyethylene glycol 600-water mixture.

ature of 30° in a constant temperature bath. 0.6-ml. quantity of 1.000 *N* sodium hydroxide solution was added, and after adequate mixing the solution was placed in the silica cuvet. The latter was transferred into the cell compartment held at constant temperature by the thermospacer assembly. Nitrogen gas, passed through water at 30° was circulated in the cell compartment to eliminate carbon dioxide absorption or oxidative changes during the experiment. The solvent cell contained solution of the surfactant at an identical concentration, to eliminate any possible ultraviolet absorptive error due to absorbance of the surfactant. The change of absorbance of the solution with time was followed at the isosbestic point and at the absorption maximum for the benzocaine, as determined on the Cary recording spectrophotometer. Application of the Beer-Lambert relation for binary mixtures was used to calculate the degree of hydrolysis. Known mixtures of benzocaine and its hydrolytic product were prepared and were found to conform to the Beer-Lambert law in the concentrations studied and in the presence of the surfactants. In those experiments where higher concentrations were utilized, silica spacers were used to reduce the path length so that the absorbance was reduced to the sensitivity range of the instrument. In all cases a minimum of a dozen readings were taken for each system. The half lives listed below were obtained from analysis of all the experimental data. Figures 3–5 include only a portion of the data in each case. The error in half-life estimation is approximately ±5% or less.

## RESULTS AND DISCUSSION

The conditions for the hydrolysis of benzocaine selected for this study were the same as were used by Higuchi and Lachman (13) in their study of the hydrolysis of benzocaine in the presence of caffeine, namely, 0.04 *N* hydroxyl ion and 30.0°. The concentration of benzocaine was 10<sup>-4</sup> *M* or less. While the overall reaction between hydroxyl ion and benzocaine is second order, the experiment

<sup>1</sup> The author wishes to acknowledge the generosity of Mr. C. D. Moore of Glover Chemicals, Ltd., of Leeds, England, for furnishing the samples of surfactants.

conditions lead to an insignificant change on the hydroxyl ion concentration. The observed reaction is, therefore, pseudo first order.

**Absorption Spectral Studies of Benzocaine in Various Surfactants.**—Figure 1 represents the ultraviolet absorption curves of *p*-aminobenzoate ion in 0.04 *N* NaOH (*B*) and of benzocaine in hexane (*A*), water (*C*), 0.53% nonionic surfactant C-14 (*D*), and in a 20% polyethylene glycol 600-water mixture (*E*). Riegelman and co-workers (15) have previously pointed out that, for certain compounds, the ultraviolet absorption spectrum of a solubilize affords a sensitive method for determining the four different modes of solubilization which have been mentioned earlier. Changes in spectrum associated with changes in the solvent may be due to (a) the influence of polarizability of the valence electrons with changes in the dielectric constant of the environment, (b) the influence of the environment on permanent or induced dipoles, (c) hydrogen bonding, dimerization, and similar molecular interactions, and (d) changes associated with variation in pH. The first two effects are associated with small changes in spectrum, whereas the last two may lead to relatively large changes in spectrum.

Relative to the single absorption band of benzocaine in water, curve *C*, the spectrum of benzocaine in hexane (*A*) illustrates the effect of changes in the polarizability of the valence electron in the molecule. The spectrum is shifted to lower wave length, possesses a larger extinction coefficient, and slightly more fine structure is noted. There appears to be three peaks, at 265 (shoulder), at 270, and at 280 (shoulder). When dissolved in surfactants or 20% polyethylene glycol 600-water mixture, the single absorption band is retained with a slight increase in the extinction coefficient, and the absorption maxima is shifted to the higher wave lengths to the same degree in each solution. From this analysis, it appears that benzocaine, when dissolved in the surfactant, is oriented in the polyoxyethylene exterior of the micelle. One may postulate somewhat on the characteristics of the polyoxyethylene exterior of a nonionic surfactant micelle. It is a well-known phenomenon that these agents in high concentrations form viscous gels wherein the water-swollen micelles are conceived as making direct contact (16). Rösch (17-20) has studied several homologous series of surfactants and found that there are approximately 2 to 2½ moles of water per oxygen atom in the surfactant. Sasaki and co-workers (21) studied the shifts in the absorption bands of selected solubilizes. For the shifts in the  $n$ ,  $\pi$ , and  $B$  absorption bands, it was concluded that surfactant micelles contain some water molecules in their exterior. Figure 2 lends further evidence to this conclusion. On the lower curve, the absorption maxima for benzocaine solutions is plotted against the log of the concentration of the added surfactant, C-14, and directly against the concentration of polyethylene glycol 600. The concentration of benzocaine was held at 1 mg. % or 10 mg. % during the study. As the concentration of the surfactant is increased, there appears to be a change in the dielectric environment surrounding the drug similar to that which takes place when benzocaine is dissolved in various concentrations of polyethylene glycol 600. Examination of the widths of the absorption curves at half density appear to indicate a negligible con-

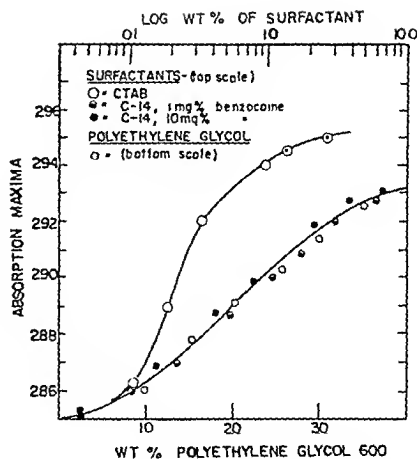


Fig. 2.—A plot showing the shift in the ultraviolet absorption maxima of benzocaine with increasing concentration of surfactant C-14 and in polyethylene glycol 600-water mixtures. The concentration of surfactants is given in log units at the top of the graph. The polyethylene glycol 600 concentration is given in linear units at the bottom of the graph.

tribution from the fraction of the benzocaine which may remain in the water phase, even at the lowest concentration studied. The shift in absorption maximum with concentration of surfactants C-30 and C-60 can be superimposed on the polyethylene glycol curve. As the concentration of surfactant is increased, the exterior of the micelle appears to undergo gradual reorientation to a closer alignment of the individual chains and, in effect, the exterior appears to take on the dielectric characteristic similar to increasing concentrations of polyethylene glycol in water.

The upper curve in Fig. 2 presents the absorption maxima for benzocaine in increasing concentrations of cetyl trimethyl ammonium bromide. It appears that there is an abrupt change in the dielectric environment surrounding the benzocaine as the micelle is formed. This would be expected if the drug is oriented near the exterior of the micelle where coulombic charge of the quaternized nitrogen group may be affecting the valence electrons of the benzocaine moiety. Similar shifts in the absorption maxima were found for sodium lauryl sulfate when the drug is in the presence of the base. The absorption curve of benzocaine in sodium lauryl sulfate solution in the absence of added base is profoundly affected by the environment and a detailed analysis of these changes and their significance will be published later.

**Hydrolysis of Benzocaine in Nonionic Surfactant Systems.**—Figure 3 and Table I present the results of the hydrolytic studies using surfactant C-14. As the concentration of the surfactant is increased, there is a gradual increase in the half life of the benzocaine under the conditions used in the study. The total concentration of benzocaine which can be incorporated into these systems is relatively large. For example, the water solubility of benzocaine is approximately 40 mg. %, while the 3.3% solution of surfactant C-14 can dissolve as much as 500 mg. %. The two upper curves of Fig. 3 represent data on 3.3% surfactant. The curve with open circles

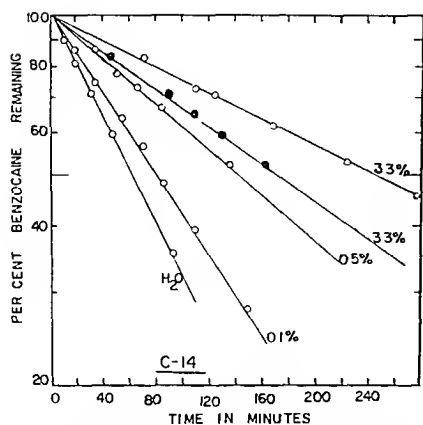


Fig. 3—A plot showing the effect of increasing concentration of surfactant C-14 on the rate of hydrolysis of benzocaine at 30° and at 0.04 *N* hydroxyl concentration. The curve with closed circles represents the data at 33 mg % benzocaine; all others at 1 mg % benzocaine.

TABLE I—INFLUENCE OF HOMOLOGOUS SERIES OF ALKALI-STABLE NONIONIC SURFACTANTS ON THE HALF LIFE OF BENZOCANE IN 0.04 *N* NaOH SOLUTIONS AT 30°

Surfactant	Half Life, min	Surfactant	Half Life, min
C 10, %		C 14, %	
0.0000	64	0.000	64
0.067	64	0.098	83
.		0.53	145
..		0.80	155
		3.3	248
		3.3 <sup>a</sup>	177
Surfactant	Half Life, min	Surfactant	Half Life, min
C-30, %		C-60, %	
0.000	64	0.000	64
0.067	64	0.067	85
1.33	153	1.33	188
3.3	239	3.3	324

<sup>a</sup> Concentration of benzocaine in this solution was 33 mg %, all others at 1 mg %.

represents the data determined when only 1 mg % of benzocaine was present in the system and the curve with closed circles includes 33 mg % of benzocaine. Both solutions resulted in pseudo first-order hydrolysis, but the thirty-three fold increase in drug concentration had a relatively small effect on the drug hydrolysis rate. When  $1/500$  of the total saturation concentration was present, the half life was 248 minutes, while when the concentration was  $33/500$  of saturation, the half life was 177 minutes. The change in drug-surfactant ratio undoubtedly influences the amount of the drug in the free state in the water environment. If a significant portion of the benzocaine were in the water phase, the absorption curve should indicate an inflection at wave lengths below the maximum relative to the placement of curves C and D in Fig. 1. It should also be detectable by comparison of the widths of the absorption curves at half density. Study of the absorption curves indicates that the drug is almost completely in the micelle, yet the half life is increased less than fourfold relative to the surfactant-free system even though the solution is  $1/500$  of satura-

tion. In accordance with the relatively high degree of hydration of the surface of the micelle, it would appear that hydrolytic attack is taking place within the micelle as well as in the aqueous phase.

Table I includes the data on the influence of the nonionic surfactant from C-10 to C-60 and on the half life of benzocaine in 0.04 *N* NaOH solution at 30.0°. The C-10 surfactant is poorly soluble in water and cannot be used in the spectrophotometric analysis procedures at higher concentrations. The apparent discrepancy in the half life with concentration of the C-14 surfactant relative to surfactant C-30 is probably due to the fact that the surfactant designated as C-14 was raised in its polyoxyethylene content by the fractional crystallization procedure required to remove the ultraviolet absorbing impurities.

There is a relatively small change in the rate of hydrolysis as one changes to a surfactant with longer polyoxyethylene chain (see Table I). It is well known that the critical micelle concentration increases with chain length, yet the hydrolysis rate appears to be affected by concentration changes to a greater extent than by changes in the hydrophilic-hydrophobic balance of the nonionic surfactant. However, different ratios of the drug to surfactant may change this conclusion.

**Hydrolysis Studies of Benzocaine in Charged Micellar Systems.**—The influence of increasing concentration of sodium lauryl sulfate on the hydrolysis rate of benzocaine is shown in Fig. 4 and in Table II. The solution containing 0.267% of sodium lauryl sulfate is approximately at the critical micelle concentration for the surfactant, 0.25% (14). The effect of the association of benzocaine close to the anionic head group of the surfactant is made a definite barrier to the approach of the hydroxyl group. The drug has been stabilized such that the half life is increased to 104 minutes. As the concentration is further increased, however, the effect is even more pronounced and the half life is extended to 1,150 minutes when the concentration is increased to 5% sodium lauryl sulfate. Under these conditions, the polar head group apparently plays a predominant role in hindering penetration of the hydroxyl group into the micelle.

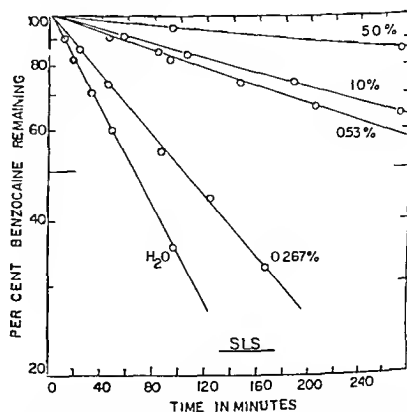


Fig. 4.—A plot showing the effect of increasing concentration of sodium lauryl sulfate on the rate of hydrolysis of benzocaine at 30° and at 0.04 *N* hydroxyl ion concentration. All curves at 1 mg % benzocaine.

TABLE II.—INFLUENCE OF ANIONIC AND CATIONIC SURFACTANTS ON THE HALF LIFE OF BENZOCAINE<sup>a</sup> IN 0.04 N NaOH SOLUTIONS AT 30°

Sodium Lauryl Sulfate, % Concentration	Half Life, min
0.000	64
0.267	100
0.533	350
1.00	420
5.00	1,150

Cetyl Trimethyl Ammonium Bromide, % Concentration	Half Life, min
0.000	64
0.067	57
1.34	425
2.46	650

<sup>a</sup> One mg % concentration in all cases

Figure 5 and Table II represents similar data of increasing concentrations of cetyl trimethyl ammonium bromide. At higher concentrations, the benzocaine appears to be shielded by the cationic polar head group. Although the negatively charged hydroxyl ion is attracted by the cationic group, it apparently cannot penetrate beyond this polar head into the deeper confines of the micelle wherein the benzocaine appears to be held. With both ionic surfactants, the predominant side for hydrolysis may be the free aqueous environment surrounding the micelles. Further work is necessary to confirm this latter postulate, however.

It should be particularly noted, however, that the lowest concentration of the quaternary studied, 0.067%, appeared to increase the hydrolysis rate rather than produce stabilization as was noted in other cases. This concentration is slightly above the CMC of 0.033% (22, 23). In a loose arrangement of chains which must be found in the initial stages of micelle formation, it is possible for the cationic head group to attract the hydroxyl ion to the environment of the benzocaine and thereby to produce an increase in the hydrolysis rate.

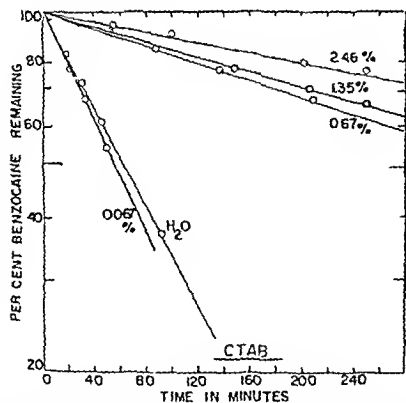


Fig 5—A plot showing the effect of increasing concentration of cetyl trimethyl ammonium bromide on the hydrolysis of benzocaine at 30° and at 0.04 N hydroxyl ion concentration

## SUMMARY

1. The surface of a micelle of nonionic surfactants appears to be relatively highly hydrated, equivalent to 5–35 per cent polyethylene glycol-water mixtures.

2. Benzocaine is solubilized in the palisade layers within the region of the hydrated polyoxyethylene chains.

3. At the drug-surfactant ratios studied, the rate of hydrolysis of benzocaine is more dependent on the concentration of the nonionic surfactant than on the length of the polyoxyethylene chain within this homologous series.

4. Hydroxyl ions apparently penetrate into the polyoxyethylene portion of the micelle, since the hydrolysis appears to be taking place in this environment as well as in the water layer.

5. In micelles composed of cationic or anionic surfactants, the polar head group apparently shields the benzocaine from direct base catalysis. These agents produce the most profound change in the hydrolysis rate extending the half life eighteen-fold in a 5 per cent solution of sodium lauryl sulfate.

6. Low concentrations of the quaternary, cetyl trimethyl ammonium bromide, just above the CMC cause a slight acceleration in the rate of hydrolysis, probably attracting the hydroxyl ion to the benzocaine environment.

## REFERENCES

- (1) Allawala, N. A., and Riegelman, S., *THIS JOURNAL*, 42, 267(1953).
- (2) Bean, H. S., and Berry, H., *J. Pharm. and Pharmacol.*, 2, 484(1950).
- (3) Bean, H. S., and Berry, H., *ibid.*, 3, 639(1951).
- (4) Patel, N. R., and Kostenbauder, H. B., *THIS JOURNAL*, 47, 289(1955).
- (5) Pisano, F. D., and Kostenbauder, H. B., *ibid.*, 48, 310(1959).
- (6) Kerm, C. J., and Antoshliw, T., *Ind. Eng. Chem.*, 42, 709(1950).
- (7) Watanabe, A., Kawazawa, T., Mima, H., Yamamoto, N., and Shima, T., *J. Pharm. Soc. Japan*, 75, 1093(1955).
- (8) Mina, H., *Yakugaku Zasshi*, 78, 983(1958), *Chem. Abstr.*, S3, 1636(1959).
- (9) Nakagawa, T., *J. Pharm. Soc. Japan*, 74, 858(1954).
- (10) Elwall, P., *Acta Chem. Scand.*, 7, 347(1953).
- (11) Allawala, N. A., and Riegelman, S., *THIS JOURNAL*, 42, 396(1953).
- (12) Sjölöf, L., *Acta Acad. Aboensis Math. et Phys.*, 20, 1(1956).
- (13) Higuchi, T., and Lachman, L., *THIS JOURNAL*, 44, 521(1955).
- (14) Miura, M., and Matsumoto, J., *J. Sci. Hiroshima Univ.*, 21, 51(1957).
- (15) Riegelman, S., Allawala, N. A., Hrenoff, M. K., and Stratt, L. A., *J. Colloid Sci.*, 13, 208(1958).
- (16) Greenwald, H. L., and Brown, G. L., *J. Phys. Chem.*, 58, 825(1954).
- (17) Rösch, M., *Kolloid Z.*, 147, 78(1956).
- (18) Rösch, M., *ibid.*, 147, 79(1956).
- (19) Rösch, M., *ibid.*, 150, 153(1957).
- (20) Rösch, M., *ibid.*, 152, 149(1957).
- (21) Sasaki, H., Okiyama, H., and Laito, S., *Bull. Chem. Soc. Japan*, 29, 752(1956).
- (22) Harley, C. S., and Runnides, D. F., *Proc. Roy. Soc. [A]*, 168, 420(1938).
- (23) Nasb, T., *J. Colloid Sci.*, 14, 59(1959).

# A New Method of Manufacture of Tablet Granulations I\*

By P. A. TUERCK, E. L. WALTERS, and E. D. CARKHUFF

A method of manufacture of tablet granulations has been developed in our laboratories which reduces granulating operations, manufacturing time, and material costs. Granulations produced by this new method have been divided into two broad classifications: low milligram potency ingredients, where a major portion of the tablets may be diluent, and high milligram potency ingredients, where active ingredients constitute a major portion of the tablet. This paper is concerned with a discussion of the low milligram potency formulations. The method involves the use of standard coating pans in which granulations are prepared by spraying with water and subsequent drying in the pan.

WHETHER THE STANDARD METHOD of manufacture be wet granulation or dry slugging with recompression, considerable time, labor, and space are involved in preparing granulations for the compressing machines. Experimental, pilot plant, and production work have been performed on a method which presents many advantages over conventional methods now employed.

In the study presented here we have attempted to describe the method and point out some of its advantages.

## EXPERIMENTAL

In the manufacture of a tablet containing a low milligram potency ingredient, the active ingredient is reduced to a fine powder by micropulverizing. This powder is introduced into a coating pan equipped with baffles and a variable speed drive. A diluent, such as powdered sugar containing 3% starch, is added. Powders are mixed by tumbling at a relatively high speed. While tumbling, the powders are sprayed with an amount of water sufficient to cause formation of small granules. Using powdered sugar as an example, this amount is equivalent to 3-4% of the total weight of the granulation. The spray must be in the form of a fine mist. As the powders are wetted, the speed of the pan is gradually reduced to prevent impaction at the periphery due to centrifugal force. Tumbling of the powders results in formation of small round granules. The speed of the pan is reduced to about 3 r. p. m. and a gentle current of hot air is blown over the tumbling granulation. In the case of powdered sugar, it is necessary to remove only about 2% moisture to obtain a granulation with satisfactory tabletting characteristics. A suitable lubricant, such as magnesium stearate, is then added, completing the operation. In practice we have noticed a small percentage of the granulations to be over 10 mesh in size. A major portion of the granulations is less than 30 mesh in size. To obviate any large lumps, the finished granulation is sifted

through an oscillating grinder equipped with a 10 mesh screen.

We have tested this method on a laboratory pilot plant, and a production scale. It is possible to manufacture and run a granulation on a compressing machine well within one hour, on an experimental or pilot plant scale, involving batches of 0.1 to 5 Kg. in size. It is possible to duplicate this operation on production batches of 200 Kg. within a period of four hours.

## APPARATUS

**Experimental Equipment.**—In preparing very small batches of about 200 Gm., a stainless steel coating pan 6.5 inches in diameter was used. The pan was mounted on a shaft driven at a constant speed of 30 r. p. m. Water was sprayed on by use of a DeVilbiss atomizer No. 127, of the type ordinarily used for nasal and throat sprays. The bulb was removed and a hose was connected to an air pressure line to provide a steady and continuous flow for uniform atomization. The expelled air tended to blow particles from the pan unless the pressure was carefully regulated to a very low pressure. After granules were formed, the granulation was dried to a moisture content suitable for satisfactory compression with a hot air gun. Due to the excessive velocity of the air output of the heat gun, sufficient drying was obtained by directing the hot air to the outside of the pan, thereby preventing the granulation from being blown out. We feel there is a great possibility that heat applied to the outside of a production size pan would be all that is needed to dry large batches of granulations with a current of air provided. This will be investigated. A hand screen of convenient size is used to sift the finished granulation. The mesh size may be 10 to 14, depending on the size of the contemplated tablet, the smaller mesh size being used for smaller tablets.

**Pilot Plant Equipment.**—A Raymond Mill was used for micropulverizing the ingredients, although any other micropulverizing apparatus would be suitable. Stainless steel or copper coating pans, 18 inches in diameter, equipped with baffles, were used. The pans were mounted, interchangeably, on a shaft attached to a d.-c. gear head motor. Speed of the pan was controlled from 0 to 30 r. p. m. by means of a Variac speed control (General Radio Co., Cambridge, Mass.). The hot air gun previ-

\* Received August 21, 1959, from the Research Laboratories of the Wm. S. Merrell Company, Cincinnati, Ohio. Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.  
The authors wish to thank Mr. Truman Sheldt for his able technical assistance in carrying out this project.

ously described was used for the drying operation. Temperature and rate of flow were controlled by a rheostat. Hand screens of suitable mesh sizes were used in the final sifting operation.

**Production Equipment.**—Ingredients were first reduced to a fine powder by passing them through a Fitzpatrick comminuter. A 48-inch baffled copper pan was provided with a variable speed drive in the range of 3 to 30 r. p. m. to accommodate production size batches of approximately 135 Kg. Batch size may be substantially increased by using larger pans. Naturally, stainless steel pans would provide obvious advantages over copper with respect to inertness, sanitation, etc. A hot air dryer was fabricated to deliver air at a temperature of 375°F. and a rate of 226 c. f. m. The temperature and rate of flow were variable. Initially, water was sprayed on the granulations by means of pneumatic guns. While such guns provide fine atomization, air pressure must be carefully regulated to prevent blowing granulations from the pans. This difficulty may be circumvented by using suitable spray nozzles which may attach directly to a water line by means of a hose and which provide satisfactory atomization with pressures of 40 to 80 psig. The dried, lubricated granulations are sifted and ground directly into storage cans through oscillating grinders equipped with suitable screens, or the finished granulations may be transferred directly to a tablet machine.

## METHOD

**Formulations.**—As a means of illustrating advantages of this new method of manufacture, a series was prepared utilizing some common diluents to prepare placebo granulations by the described method as well as by a conventional method in a Day mixer.

A series of eight experimental granulations were prepared by spraying the powders described below with water. A water-soluble dye, D&C Green No. 5, was first mixed with each powder in a concentration of 0.5 Gm. per kilogram and micropulverized in a Raymond mill before granulating. This color was chosen because it usually causes difficulty in producing smoothly colored tablets when granulated by ordinary wet granulating methods. In all cases batch sizes of 2.5 Kg. were used as a matter of convenience for processing in existing pilot plant equipment. The wet granulations thus formed were dried while tumbling in the coating pan, lubricated with 1% magnesium stearate, and compressed on a model RB-2 Stokes single rotary tableting machine using  $1\frac{1}{32}$  inch standard cup punches and dies at a tablet weight of 400 mg.

In like manner the series was repeated, granulations being made in a small Day mixer by adding water as a granulating agent. Wet granulations were forced through a 6-mesh screen and dried overnight on trays in a hot air oven at 120°F. Dried granulations were ground through a 12-mesh screen, lubricated, and compressed as previously described.

Materials comprising the series are as follows: (A) powdered sugar; (B) powdered sugar 90%, corn starch 10%; (C) lactose; (D) lactose 90%, corn starch 10%; (E) mannitol 98%, powdered gelatin 2%; (F) mannitol 88%, powdered gelatin

2%, corn starch 10%; (G) powdered sugar 50%, lactose 25%, corn starch 25%; (H) powdered sugar 90%, corn syrup solids 10%.

Table I compares granulations and tablets obtained by both methods with respect to moisture content, drying times, mesh sizes, compression characteristics, hardness, disintegration, and general appearance. Advantages of the spray granulation method over the conventional method described are evident in this table. It is interesting to note that in spite of a high percentage of "fines" in some instances (spray granulations C, D, E, F), excellent tableting characteristics were maintained.

In the interests of simplicity, stability, and economy, a plain powdered-sugar base is satisfactory for the majority of low milligram potency medicaments.

Examples of formulations successfully prepared with a sugar base are phenobarbital tablets (15 mg., 30 mg., 60 mg., 90 mg.), thiamine hydrochloride tablets (10 mg., 25 mg., 50 mg., 100 mg.), ascorbic acid tablets (50 mg., 100 mg.), and a variety of specialty products.

**Coloring.**—Granulations may be colored simply by micropulverizing pigments, lakes, or dyes along with the other tablet ingredients as they are being introduced in the pan. Although water-soluble dyes may be distributed on a portion of the diluent and dried prior to grinding, we have found that even this step may be eliminated by using dyes soluble in volatile solvents, such as alcohol or chloroform, and merely pouring them over portions of the sugar prior to micropulverizing. Naturally, the micropulverizing equipment must be hooded and ventilated to eliminate explosion or health hazards. Any residual solvent is driven off during the drying operation in the coating-pan.

**Particle Size.**—The mesh size of the final granulation may be controlled by regulating the amount of moisture sprayed on the granulation and by the length of time the moist granulation is tumbled in the coating pan.

If the granulation is overwet or tumbled too long, large balls result. If underwet, the granulation may have a very large proportion of "fines."

When properly made, granulations prepared by this method lend themselves admirably to high speed tableting operations. Characteristics of the granulations are such that embossing or bisecting present no problems.

**Lubrication.**—A variety of lubricants have been used. Magnesium stearate was satisfactory, although other stearates and talc have been used. After introduction of the lubricant to the batch, a final spraying of 0.25 to 0.5% of light mineral oil has been found to eliminate further dusting and to provide a gloss to the tablet.

**Processing Time.**—As previously mentioned, a 135-Kg. batch of granulation may be completed to the point of compressing within a three or four-hour period. Spraying time may be accomplished within ten minutes; drying time within one or two hours. The major portion of manufacturing time is required for the manual charging of the pan and removal of the finished granulation from the pan. Refinements are possible to shorten these times further. By increasing the temperature of the air delivered by the drying gun, the drying

TABLE I — COMPARISON OF GRANULATIONS AND TABLETS MADE BY THE SPRAY GRANULATING METHOD WITH THOSE PREPARED IN A DAY MIXER

		Granulations										Tablets					
Method		Granulating Solution Used (Water), ml	% Moisture in Wet Granulation (Karl Fischer)	Drying Times hr	% Moisture in Dry Granulation (Karl Fischer)	Classification of Screen Mesh Sizes of Dry, Ground Granulations, %							Compression Characteristics <sup>a</sup>	Hardness (Monsanto)	Disintegration U. S. Method, Min 37° Water	General Appearance <sup>b</sup>	
						20	30	40	50	100	200	<200					
A	Spray	120	3 06	0 25	0 42	22	13	15	13	17	12	8	1	9	5	1	
A	Day mixer	185	6 9	18	0 53	52	13	9	8	10	6	2	2	8	5	4	
B	Spray	125	5 17	0 17	1 02	14	8	10	12	20	17	19	1	7 5	3	1	
B	Day mixer	200	8 7	18	1 02	58	12	8	6	11	4	1	2	7	9 5	4	
C	Spray	250	11 48	0 17	5 47	20	5	3	3	3	10	56	1	9 5	10	1	
C	Day mixer	365	18 68	18	4 71	47	14	9	7	10	5	8	3	5	15	4	
D	Spray	275	11 31	0 17	6 14	9	3	3	2	5	15	63	1	9	1 5	1	
D	Day mixer	535	22 04	18	4 66	59	15	9	6	7	3	1	2	7	1	4	
E	Spray	250	6 53	0 5	0 48	41	9	5	5	9	7	24	1	12 5	9	2	
E	Day mixer	675	20 6	18	0 48	46	14	10	8	11	5	6	2	11 5	9	4	
F	Spray	275	7 11	0 5	1 33	12	4	3	2	10	14	55	1	9 5	0 75	1	
F	Day mixer	800	25 83	18	0 51	60	12	6	4	9	8	1	2	9 5	6	4	
G	Spray	215	9 36	0 5	2 02	12	5	7	8	23	28	17	1	9	3	2	
G	Day mixer	260	11 7	18	5 19	26	10	10	12	29	12	1	2	7	3	4	
H	Spray	100	3 38	0 25	0 53	5	4	7	12	46	22	4	1	9 5	5 5	3	
H	Day mixer	165	6 73	18	1 12	63	15	8	5	5	3	1	2	7 5	7 5	4	

<sup>a</sup> 1, good, 2, fair, 3, poor (capping)<sup>b</sup> 1, Excellent, 2, good, 3, fair (slightly mottled), 4, poor (badly mottled and spotted)

TABLE II — A COMPARISON OF MANUFACTURING TIMES REQUIRED FOR PRODUCTION SIZE BATCHES OF TABLET GRANULATIONS MADE IN A DAY MIXER AND BY THE SPRAY TECHNIQUE

	Phenobarbital, 15 mg				Thiamine HCl, 10 mg				Ascorbic Acid, 50 mg			
	Day Mixer	Total	Spray Method	Total	Day Mixer	Total	Spray Method	Total	Day Mixer	Total	Spray Method	Total
	Labor Time, hr	Mfg Time, hr	Labor Time, hr	Mfg Time, hr	Labor Time, hr	Mfg Time, hr	Labor Time, hr	Mfg Time, hr	Labor Time, hr	Mfg Time, hr	Labor Time, hr	Mfg Time, hr
Loading and granulating	1 5	1 5	0 5	0 5	0 75	0 75	0 25	0 25	1 0	1 0	0 4	0 4
Wet screening	1 75	1 75			1 0	1 0			1 0	1 0		
Drying		16		2		15		1 25		16		1 5
Dry screening	1 0	1 0	0 5	0 5	1 0	1 0	0 25	0 25	0 25	0 25	0 3	0 3
Lubrication	0 75	0 75	0 1	0 1	0 5	0 5	0 1	0 1	0 25	0 25	0 1	0 1
Total cleanup	0 75	0 75	0 25	0 25	1 0	1 0	0 25	0 25	0 5	0 5	0 25	0 25
Total	5 75	21 75	1 35	3 35	4 25	20 25	0 85	2 1	3	19	1 05	2 55
Labor time saved			4 4				3 4				1 95	
Production time saved				18 4				18 15				16 45
Batch size		2,750,000				300,000				400,000		
Total wt of material		214 Kg				89 5 Kg				112 5 Kg		

cycle may be further shortened. When a high level of starch is used, more water is needed for granulation and the drying time is slightly longer. However, with regard to compression characteristics, the amount of moisture is not as critical as in a straight sugar granulation.

As a means of illustration, Table II emphasizes savings effected in production time on three typical U S P tablet granulations. Comparisons are drawn between batches made on a production scale by a conventional wet granulating method in a Day mixer and batches made in a coating pan using the spray technique. It must be realized that methods of granulation and production techniques will vary considerably from company to company. Data contained in Table II are, therefore, relative only to our particular production methods.

**Stability.**—A considerable number of tablet granulations are currently manufactured which, because of heat and moisture lability, require protracted drying times in dehumidified areas to

preserve stability. Many such granulations may be safely made by our method, since a smaller amount of moisture is introduced into the granulation, and since it is dried to a satisfactory level for compressing within a short period of time. Although high temperatures (160–500°F.) may be used in the drying operation, our experience has been that the temperature within the granulation is maintained at safe limits, because of the rate of evaporation of the granulating agent, until the granulations begin to dry. Naturally, a close check must be kept on moisture content to prevent overdrying with a resultant increase in temperature. Furthermore, if an anhydrous base is required, tablets may be packaged immediately with silica gel packets for further moisture reduction.

**Tabletting.**—Granulations prepared as described may be run at high speeds. Due to the soft nature of the granulations, wear on punches, dies, and other machinery is reduced to a minimum.

**Disintegration.**—Tablets prepared by the outlined

method dissolve rather than disintegrate. The solution or disintegration time has not been found to exceed fifteen minutes when tested by the U. S. P. XV method.

### CONCLUSIONS

1. A new method for manufacturing granulations of low milligram potency medicaments has been developed.

2. The method involves the use of standard coating pans in which granulations are formed by spraying with water and subsequent drying in the pan.

3. The method has advantages of decreasing material costs, reducing manufacturing time, and eliminating a number of processing steps, resulting in economies in labor and space.

---

## A New Method of Manufacture of Tablet Granulations II\*

By P. A. TUERCK, E. L. WALTERS, and E. D. CARKHUFF

This paper is concerned with a discussion of a method of manufacture for tablet granulations in which active ingredients comprise a major portion of the tablet. The method involves the use of standard coating pans in which granulations are formed by spraying with starch paste or other commonly used granulating solutions and subsequent drying in the pan.

IN SOME INSTANCES, due to limitations in space and because rapid disintegration is required, a modification of the granulating method described in Part I is desired.

The method described in Part I principally utilizes sugar and other water-soluble ingredients as diluents. Tablets resulting from such granulations dissolve rather than disintegrate.

We have pointed out in the description of the method of manufacture that granulations containing major proportions of sugar and other water-soluble ingredients may be made by spraying with water and drying while the coating pan is continuously rotated. This is possible because of the durability of the granulation formed.

We have found that in instances where active ingredients form a major portion of the tablet and where rapid disintegration is desired (A. P. C. tablets, for example), it is desirable to spray granulate with starch paste. In other formulations, gelatin, glucosc, sorbo, and other granulating agents may be preferred.

In some instances, particularly when starch paste is the granulating agent, we have noticed that, when the procedure outlined in Part I was followed, a satisfactory granulation could not be obtained. This result may be attributed to the fact that starch granulations are extremely soft until dry. The continuous tumbling action in the coating pan reduced the wet granulation to a fine powder. A further complicating factor was the necessary use of a considerably larger amount of granulating agent in this type of formulation which increased drying time and, hence, aggravated the formation of "fines."

An interesting modification of the procedure is also being investigated wherein dry soluble starch or other dry gums are mixed with the powdered ingredients before granulating. By spraying the granulation with water, starch paste or gum solutions are produced *in situ*. This expedient permits granulation with lesser amounts of moisture and is particularly effective in maintaining smoothness of color when a colored tablet is desired.

In like manner, dry ethyl cellulose may be mixed with ingredients and the granulating solution produced *in situ* by spraying with ethyl or isopropyl alcohol.

### APPARATUS

To circumvent the difficulty, two changes were made in the equipment. First, the hot air source was modified so that the temperature of delivered air was 400-600°F. This experiment shortened the drying time by increasing the rate of evaporation. Secondly, a timing mechanism was placed on the motor

\* Received August 21, 1959, from the Research Laboratories of The Wm. S. Merrell Co., Cincinnati, Ohio.

Presented to the Scientific Section A. P. C., Cincinnati meeting, August 1959.

The authors wish to thank Mr. Truman Sheldt for his able technical assistance in carrying out this project.



of the pan which automatically rotated the pan one revolution every minute. This was put in operation only during the drying cycle. Depending upon the drying characteristics of different granulations, the intervals at which the pan is rotated may be easily changed by adjusting the timing mechanism.

This expedient allowed the exposed surface to dry during each resting cycle. The time the granulation was in motion was greatly reduced, thereby eliminating formation of too many "fines."

With these exceptions, equipment and procedure is the same as outlined in Part I.

### EXPERIMENTAL

Granulations (A, B, and C) of high milligram potency materials were prepared by both the spray granulation technique and by a conventional method of wet granulating in a Day mixer as illustrated below. The granulations as well as tablets prepared from them are compared in Table I.

GRANULATION A—A. P. C.

	Day Mixer Method, Kg.	Spray Method, Kg.
Acetophenetidin	2 52	2 52
Caffeine	0 5	0 5
Corn starch	0 72	0
Soluble starch (Corn Products Refining Co.)	0	0 72
Add at time of lubrication:		
Aspirin granulation (16% starch)	4 6	4 6
Corn starch q. s. to	8 7	8 7

**Day Mixer Method.**—Mix and granulate ingredients with a solution composed of 10% starch paste and 16% gelatin. Wet screen through a 6-mesh screen. Spread on trays and dry overnight at 120°F. Dry screen through a 12-mesh screen. Add aspirin granulation and corn starch lubricant. Compress on  $\frac{7}{16}$  inch standard cup punches at a weight of 518 mg.

**Spray Method.**—Mix ingredients in a coating pan and granulate by spraying with water. Dry, add aspirin granulation, and lubricant to pan. Sift through 10-mesh screen. Compress as above.

GRANULATION B—SULFADIAZINE, 500 MG. PER TABLET

	Day Mixer Method, Kg.	Spray Method, Kg.
Sulfadiazine	3	3
Corn starch	0 34	0 3
Corn syrup solids	0	0 075
Soluble starch (Corn Products Refining Co.)	0	0 15

**Day Mixer Method.**—Mix and granulate with a solution composed of 5% starch paste and 5% glucose. Wet screen through a 6-mesh screen. Dry overnight on trays at 120°F. Dry screen through a 12-mesh screen. Lubricate with 1% magnesium stearate and compress at 616 mg., using  $\frac{7}{16}$  inch standard cup punches.

**Spray Method.**—Mix ingredients in coating pan. Granulate by spraying with water. Dry and lubricate in the pan. Compress as above.

GRANULATION C—ASCORBIC ACID, 250 MG. PER TABLET

	Day Mixer Method, Kg.	Spray Method, Kg.
Ascorbic acid, powdered	1 605	1 605
Sugar, powdered	0 54	0 54
Lactose	0 816	0 816
Acacia, U. S. P. white powder	0 018	0 018
Corn starch	0 093	0
Soluble starch (Corn Products Refining Co.)	0	0 093

**Day Mixer Method.**—Mix and granulate ingredients with 16% gelatin solution. Wet screen through a 6-mesh screen. Spread on trays and dry overnight at 120°F. Dry screen through a 12-mesh screen. Lubricate with 1% magnesium stearate. Compress on  $\frac{7}{16}$  inch standard cup punches at a weight of 544 mg.

**Spray Method.**—Mix ingredients in a coating pan and granulate by spraying with water. Dry and lubricate in the pan and compress as above.

### FORMULATIONS

Although a major portion of our work relating to the high milligram potency type of granulation has been with a starch paste granulating agent, instances have arisen where modifications were necessary.

Some water-insoluble materials, particularly when fluffy or crystalline in nature, require other granulating agents. In some instances granulation may be accomplished by merely spraying with a volatile solvent for the material in question. In other cases it is necessary to add additional binders, such as ethyl cellulose, methyl cellulose, polyvinylpyrrolidone etc., to the solvent before spraying. Although the cost of the solvents presents a disadvantage, drying time is considerably shortened.

As might be expected, care must be taken in proper selection of disintegrating agents, each granulation presenting its individual problem.

### PROCESSING TIME

Since starch paste granulations require substantially more granulating agent for satisfactory results we have found it necessary to use longer periods of time for the drying operation. Drying may be accomplished overnight by the method described. In practice, we have found that for scheduling purposes it is more convenient to manufacture the sugar base or fast drying granulations during the day and to spray the slower drying starch paste granulations near the end of the day's operations, thereby allowing an overnight period for drying. We are currently investigating refinements which will shorten drying time.

Table II illustrates the difference in manufacturing time required on production size batches when the spray method is compared with wet granulation in a Day mixer. Economies in both labor and total manufacturing time are effected in the spray method.

TABLE I.—COMPARISON OF GRANULATIONS AND TABLETS MADE BY THE SPRAY GRANULATING METHOD WITH THOSE PREPARED IN A DAY MIXER

Method	Granulating Solution Used, ml	Granulations				Tablets									
		Moisture in, % Wet Granulation,	Drying Time, hr	Moisture in % Dry Granulation,	Classification of Screen mesh Sizes of Dry, Ground Granulations, %	Compression Characteristics <sup>a</sup>	Hardness (Monsanto)	Disintegration, U S P Method, 37°, Water, min	General Appearance <sup>c</sup>						
20	30	40	50	100	200	<200									
A Spray	450 <sup>a</sup>	9.4 <sup>c</sup>	0.5	2.19 <sup>c</sup>	33	12	8	3	16	20	8	1	10	0.25	1
A Day mixer	1,600 <sup>b</sup>	26.4 <sup>c</sup>	18	1.91 <sup>c</sup>	60	18	9	4	4	3	2	1	8.5	0.7	2
B Spray	1,200 <sup>a</sup>	22.4 <sup>c</sup>	0.7	1.8 <sup>c</sup>	5	2	3	7	52	27	4	1	13	15	1
B Day mixer	1,600 <sup>b</sup>	22.4 <sup>c</sup>	18	1.4 <sup>c</sup>	12	10	10	10	22	23	13	1	13	13	1
C Spray	200 <sup>a</sup>	6 <sup>d</sup>	0.25	3.8 <sup>d</sup>	22	10	9	8	21	17	13	1	12	6	1
C Day mixer	225 <sup>b</sup>	8.5 <sup>d</sup>	18	4 <sup>d</sup>	60	15	10	6	6	2	1	2	9	8	2

<sup>a</sup> Water  
<sup>b</sup> Refer to formula  
<sup>c</sup> Karl Fischer.  
<sup>d</sup> Twenty-four hour drying at 100°  
<sup>e</sup> 1, Good, 2, capping tendency  
/ 1, Good, 2, spotty, chipping tendency on edges

TABLE II.—A COMPARISON OF MANUFACTURING TIMES REQUIRED FOR PRODUCTION SIZE BATCHES OF TABLET GRANULATIONS MADE IN A DAY MIXER AND BY THE SPRAY METHOD

	A P C				Sulfadiazine, 500 mg				Ascorbic Acid, 250 mg			
	Day Mixer	Spray Method	Day Mixer	Spray Method	Day Mixer	Spray Method	Day Mixer	Spray Method	Day Mixer	Spray Method	Day Mixer	Spray Method
	Labor Time, hr	Total Mfg Time, hr	Labor Time, hr	Total Mfg Time, hr	Labor Time, hr	Total Mfg Time, hr	Labor Time, hr	Total Mfg Time, hr	Labor Time, hr	Total Mfg Time, hr	Labor Time, hr	Total Mfg Time, hr
Loading and granulating	1.5	1.5	0.5	0.5	1.5	1.5	0.4	0.4	1.0	1.0	0.4	0.4
Wet screening	2.0	2.0			2.25	2.25			1.5	1.5		
Drying		16		2.0		16		1.5		16		1.5
Dry screening	1.0	1.0	0.4	0.4	1.5	1.5	0.3	0.3	0.5	0.5	0.3	0.3
Lubrication	1.25	1.25	0.5	0.5	0.25	0.25	0.1	0.1	0.25	0.25	0.1	0.1
Total clean-up	0.75	0.75	0.25	0.25	1.0	1.0	0.25	0.25	0.25	0.25	0.25	0.25
Total	6.5	22.5	1.65	3.65	6.5	22.5	1.05	2.55	3.5	19.5	1.05	2.55
Labor time saved			4.85				5.45				2.45	
Production time saved				18.85				19.95				16.95
Batch size		840,000				200,000				200,000		
Total wt of material, Kg		435				123				109		

by eliminating certain steps in manufacture, cutting down on clean-up time, and shortening drying time .

Coloring, granulating, lubricating, and tableting characteristics of the granulations discussed may be considered to be substantially the same as those discussed under Part I

The wear on punches, dies, and machinery depends to a large extent on the abrasiveness and physical characteristics of the active ingredient We have found that our method has advantages over standard methods, particularly when a binder is present in the granulating solution Since, by our method, the granulating agent is sprayed on the ingredients, they tend to be coated with the binding agent, thereby minimizing the abrasiveness or gumminess of the granules We have prepared granulations which caused binding when standard techniques were used, but which compressed with no difficulty when spray granulating was employed.

DISINTEGRATION AND STABILITY

Tablets made by the method outlined in Part II usually disintegrate rather than dissolve.

Since there is no major change in diluents by this method, stability of tablets is comparable to that of tablets made by standard procedures

CONCLUSIONS

- 1 A new method for manufacturing granulations of high milligram potency medicaments has been developed
- 2 The method involves the use of standard coating pans in which granulations are formed by spraying with starch paste or other commonly used granulating solutions, with subsequent drying in the pan

# Esterification of Two Sterically Hindered Acids Using Ultrasound Waves\*

By ROBERT J. GERRAUGHTY† and PAUL J. JANNKE

The possibility of using ultrasound waves to effect the direct esterification of sterically hindered acids was explored. The methyl, ethyl, *n*-propyl, and *n*-butyl esters of *o*-thymotic and ursolic acids were prepared by the application of low frequency ultrasound waves, the rates of esterification were determined, and the mechanism of the reaction was studied, using methyl mercaptan in place of an alcohol. Branched chain esters of the acids could not be prepared even when exposure conditions were broadly varied.

NUMEROUS STUDIES have been conducted on the direct esterification of sterically hindered acids. The success of the reaction varies over a broad range, depending, in part, upon the structures involved and the conditions applied. Nevertheless, esters of many of these acids have been prepared, frequently by indirect methods. The application of ultrasound waves to overcome steric hindrance was deemed advisable because it has been shown that such energy is capable of stimulating a wide variety of chemical reactions, some of them involving factors associated with esterification.

The kind of energy used is of utmost importance because high frequencies and cavitation favor the hydrolysis of esters (1-4); therefore it appeared logical to assume that low frequencies and lessened cavitation might favor ester formation.

This study employed two acids, one of which is strongly hindered, the other being more moderately so. *o*-Thymotic acid is strongly hindered because of substituents occupying both positions *ortho* to the carboxyl group. Attempted condensations of it with alcohols in the presence of strong dehydrating agents lead to the formation of thymotides (5) instead of the conventional esters. Ursolic acid, which is a pentacyclic alicyclic acid, shows steric hindrance because of the spatial orientation of the cyclic system adjacent to the ring which carries the carboxyl group (6).

A method for the esterification of the two acids is described and the results are shown in Tables I and II. Rates of esterification of both acids

were determined. Table III indicates that esterification of ursolic acid is gradual at first, reaches a maximum after seventy-five minutes, and declines rapidly after ninety minutes. *o*-Thymotic acid (Table IV) also is esterified slowly at first, reaching a maximum rate after one hundred and five minutes, and then declining rapidly.

A study of the mechanism of the reaction was conducted to determine if ultrasound altered it in any way. Under normal conditions in direct esterification, the acid acts as a hydroxyl donor and the alcohol as a hydrogen donor (7). This appears not to be the case when esterification is accomplished under the influence of ultrasound. The condensation of the acids with methyl mercaptan proceeded in such a manner as to result in the formation of hydrogen sulfide, indicating clearly that under these conditions the acids served as hydrogen donors, otherwise water would have been formed.

## EXPERIMENTAL

**Preparation of Ursolic Acid Esters.**—Fifty milliliters of the selected alcohol was degassed by exposure<sup>1</sup> at 80 to 85 ma. plate current and 39.1 kc. for one hour. One gram of the ursolic acid, 2 drops of concentrated sulfuric acid catalyst, and 2 Gm. of anhydrous copper sulfate (to remove water formed in the reaction) were added to the degassed alcohol. The polyethylene bottle used as the exposure vessel was immersed below the surface of the water in the transducer tank to decrease cavitation, and the mixture was exposed at not more than 45 ma. plate current for two hours, and then filtered. The solvent was evaporated and the residue was dissolved in ether. The ethereal solution was washed with three successive 100-ml. portions of 3% sodium hydroxide solution and then washed twice with 100-ml. quantities of distilled water. The ether was evaporated and white crystalline ursolic acid ester was recrystallized twice from 70% ethanol. Straight chain esters were prepared by this method in good yields (Table I).

Branched chain esters could not be prepared even by extending exposure periods or increasing the plate current.

Carbon-hydrogen determinations were made according to the method of Niederl and Niederl (8).

Preparation of acetyl derivatives of the esters by refluxing with acetic anhydride for one hour yielded acetyl ursolic acid esters that compared favorably with those reported in the literature, as shown in Table I.

<sup>1</sup> "Sonogen" Ultrasonic Generator, Model 25-B, Branson Ultrasonic Corp., Stamford, Conn.

\* Received August 21, 1959, from the School of Pharmacy, University of Connecticut, Storrs.

Abstracted from a thesis submitted to the Graduate School of the University of Connecticut by Robert J. Gerraughty in partial fulfillment of the requirements for the Doctor of Philosophy degree.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: College of Pharmacy, Rutgers—The State University, 1 Lincoln Ave., Newark 4, N. J.

TABLE I—URSOLIC ACID ESTERS

Ester	Yield <sup>a</sup>	Melting Point, ° C		Melting Point Acetyl Esters, ° C		Carbon and Hydrogen Analysis, %			
		Reported (10)	Found	Reported (11)	Found	Calcd.		Found <sup>b</sup>	
						C	H	C	H
Methyl	80.1	169-170	170-171	245	243-244	79.15	10.64	79.0	10.7
Ethyl	67.1	.	198-199	194	192-193	79.32	10.74	79.1	10.6
<i>n</i> -Propyl	47.9	.	190-191	173	171-172	79.52	10.84	79.4	10.8
<i>n</i> -Butyl	41.0	.	180-182	123-125	124-126	79.69	10.94	79.7	10.9

<sup>a</sup> Average of two determinations    <sup>b</sup> Average of three determinationsTABLE II—*o*-THYMOTIC ACID ESTERS

Ester	Yield <sup>a</sup>	Boiling Point, ° C		Saponification Equivalent <sup>b</sup>		Carbon and Hydrogen Analysis, %			
		Reported (12)	Found	Calcd	Found <sup>c</sup>	Calcd		Found <sup>d</sup>	
						C	H	C	H
Methyl	49.5	142/18 5 mm.	143/20 mm.	538	532	69.23	7.21	69.3	7.0
Ethyl	53.9	153/18 5 mm.	155/20 mm.	505	494	70.27	7.66	70.6	7.6
<i>n</i> -Propyl	50.3		165/20 mm.	475	464	71.19	8.05	71.4	8.0
<i>n</i> -Butyl	43.5		175/20 mm.	452	442	72.00	8.40	71.8	8.3

<sup>a</sup> Average of two determinations    <sup>b</sup> Expressed in mg. of potassium hydroxide    <sup>c</sup> Average of two determinations    <sup>d</sup> Average of two determinations

TABLE III.—RATES OF ESTERIFICATION OF URSOLIC ACID ESTERS

Time of Exposure, min	Methyl	Ethyl	<i>n</i> -Propyl	<i>n</i> -Butyl
0	0.0	0.0	0.0	0.0
15	1.2	0.0	0.0	0.4
30	4.3	0.6	2.5	0.4
45	10.5	1.9	3.8	4.7
60	16.6	7.2	6.4	6.1
75	35.2	20.4	15.5	14.5
90	69.1	45.6	35.0	34.1
105	76.8	57.6	42.8	39.7
120	78.3	61.6	45.4	43.9
135	79.9	64.2	48.0	48.1

TABLE IV.—RATES OF ESTERIFICATION OF *o*-THYMOTIC ACID ESTERS

Time of Exposure, min	Methyl	Ethyl	<i>n</i> -Propyl	<i>n</i> -Butyl
0	0.0	0.0	0.0	0.0
15	0.0	0.0	0.0	0.0
30	0.3	0.2	0.6	0.0
45	6.3	3.9	5.1	2.9
60	12.3	12.1	10.3	9.4
75	20.3	21.0	17.1	16.1
90	30.2	30.7	27.5	24.1
105	44.2	45.6	43.9	38.6
120	48.8	47.9	46.9	44.5
135	50.8	49.4	46.9	45.9
150	51.5	50.1	47.7	45.9

Attempts to determine saponification equivalents of the esters were unsuccessful due to the resistance of these esters to even the most drastic conditions for hydrolysis.

Preparation of *o*-Thymotic Acid Esters.—The same method that was used to prepare ursolic acid esters was followed and the results were comparable, except that when the solvent was evaporated, the residue was liquid in all cases. These liquid esters were dissolved in ether and washed with 3% sodium

hydroxide solution followed by washing with dilute hydrochloric acid solution. The ether was evaporated on a water bath and the remaining liquid was distilled *in vacuo*. The esters were colorless when distilled, but changed to pale yellow immediately afterward. Fractional distillation on a Todd column<sup>2</sup> failed to remove the yellow color. Again, only the straight chain esters could be synthesized (Table II). Carbon-hydrogen results and saponification equivalents by a method using diethylene glycol as the solvent (9) are shown also in this table.

Rates of Esterification of Ursolic Acid Esters.—The rate of esterification for each of the esters was determined under the same conditions that were used for their preparation. Weighed quantities of ursolic acid were exposed, using the proper alcohols, and aliquots were extracted at fifteen-minute intervals. These aliquots were titrated with standard sodium methoxide solution using thymol blue as the indicator. Sodium methoxide solution was used because reaction was too slow with other alkali solutions. The percentage of unesterified acid was calculated and the per cent of esterification was then apparent by difference. The results are shown in Table III.

Rates of Esterification of *o*-Thymotic Acid Esters.—The rate of esterification of each of the four esters was determined under the same conditions that were used for their preparation. Weighed quantities of *o*-thymotic acid were exposed, using the proper alcohols, and aliquot portions were extracted at fifteen-minute intervals and titrated with standard sodium hydroxide solution, using phenolphthalein as the indicator. The percentage of unesterified acid was calculated, and the per cent of esterification was then apparent by difference. The results of this study are given in Table IV.

Mechanism of Esterification Using Ultrasound.—One gram of ursolic acid was suspended in 100 ml. of ethanol. Two grams of methyl mercaptan and a drop of concentrated sulfuric acid were added. The mixture was exposed at 39.1 kc. and 45 ma.

<sup>2</sup> Todd Precision Fractionation Assembly, Todd Scientific Co., Springfield, Pa.

plate current for two hours. The odor of hydrogen sulfide was apparent after about one hour of the exposure. The solution was poured into water, and the white precipitate which formed was collected on a sintered-glass funnel. The compound was purified in the same manner as stated previously, yielding 0.452 Gm (43.9% of theoretical) of methyl ursolate, m p 168–170°. After a sodium fusion, no positive test for sulfur was obtained. A mixed melting point with a genuine sample of methyl ursolate gave no appreciable lowering of the melting point. The indication is that the acid acted as a hydrogen donor and not a hydroxyl donor in this reaction. Reaction of ursolic acid with ethyl mercaptan by the same procedure produced ethyl ursolate, m p 197–199°, further verifying the mechanism involved.

### SUMMARY AND CONCLUSIONS

Low frequency ultrasound waves have been demonstrated to be effective in facilitating the direct esterification of *o*-thymotic and ursolic acids, both of which are sterically hindered.

Straight chain esters of the acids, using alcohols having up to four carbon atoms, were prepared. Branched chain esters of these acids could not be prepared even after prolonged periods of ex-

posure and increased plate current. This is believed to be due to the bulk of the entering group.

Data showing the rates of esterification of the acids are reported.

The mechanism of the reaction using ultrasound was established to be the donation of hydrogen by the carboxyl group rather than the donation of hydrogen by the alcohol.

### REFERENCES

- (1) Renaud, L., and Renaud, P., *J chim phys*, 49, 644 (1952).
- (2) Gray, W., *Dissertation Abstr*, 12, 462 (1952).
- (3) Mastagli, P., and Mahoux, A., *Compt rend*, 228, 684 (1949).
- (4) Miyagawa, I., *J Soc Org Synthet Chem Japan*, 7, 167 (1949).
- (5) Spallino, R., and Provenzal, G., *Gazz chim ital*, 39, 325 (1909).
- (6) Eisen, H., *Drug Standards*, 23, 186 (1955).
- (7) Roberts, I., and Urey, H., *J Am Chem Soc*, 61, 2584 (1939).
- (8) Niederl, J., and Niederl, V., "Micromethods of Quantitative Organic Analysis," 2nd Ed., John Wiley & Sons, Inc., New York, N. Y., 1942, pp 101–131.
- (9) Shriner, R., and Fuson, R., "The Systematic Identification of Organic Compounds," 3rd Ed., John Wiley & Sons, Inc., New York, N. Y., 1948, pp 133–134.
- (10) Sell, H., and Kremers, R., *J Biol Chem*, 126, 501 (1938).
- (11) *Ibid*, 125, 451 (1938).
- (12) Spallino, R., and Provenzal, G., *op cit*.

## Studies on the Stability of Filipin I\*

### Thermal Degradation in the Presence of Air

By JAMES E. TINGSTAD and EDWARD R. GARRETT

The loss of biological activity of crystalline filipin has been statistically correlated with the loss of spectrophotometric absorbance at 355  $m\mu$ . Filipin is oxidatively destroyed in air by an apparent second-order reaction similar to the one involved in the aerobic degradation of fumagillin. The degradation product(s) interfere slightly with the U. V. assay, and correction factors are applied. The rate constants for the reaction at 70, 60, 50, and 37° have been evaluated and the apparent heat of activation calculated. Half lives at 30, 25, and 4° have been estimated. Preliminary experiments show that filipin is at least 50 to 100 times more stable under nitrogen than in air. A new empirical formula,  $C_{32}H_{54}O_{10}$ , determined on especially purified filipin is given.

FILIPIN, A POTENT antifungal agent isolated from *Streptomyces filipinensis* by Whitfield and co-workers (1), is a neutral conjugated polyene with an empirical formula of  $C_{32}H_{54}O_{10}$ .<sup>1</sup>

Filipin strongly absorbs ultraviolet light, with maxima at 322, 338, and 355  $m\mu$  (Fig 1, curve

A). Whitfield and associates reported (1) that filipin is susceptible to oxidation and that the loss of absorbance at 338 and 355  $m\mu$  closely parallels the loss of biological activity. This is to be expected since its general structure resembles that of another polyene antibiotic, fumagillin; the degradation of which was studied by Garrett and Eble (2, 3, 4).

In order to determine the normal shelf life of the drug if protected from light, the thermal, aerobic, nonphotolytic degradation of crystalline

\* Received August 21, 1959, from the Upjohn Co., Kalamazoo, Mich.  
Presented to the Scientific Section, A Ph A, Cincinnati meeting, August 1959.

<sup>1</sup> In a previous publication (1) the empirical formula was given as  $C_{32}H_{54}O_{10}$ . Recent analyses, by W. A. Struck and associates of these laboratories, of material vigorously purified by G. B. Whitfield, also of these laboratories, confirm the new formula.

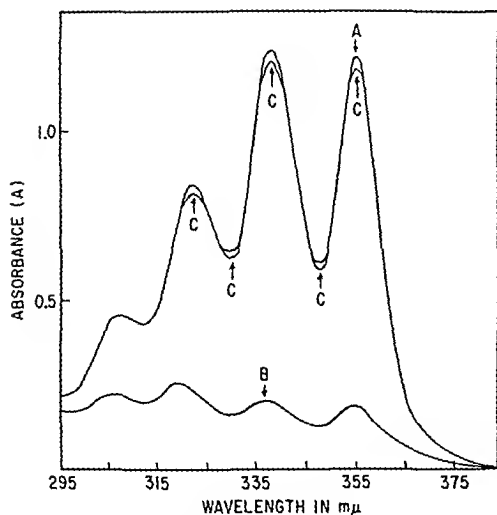


Fig. 1.—Ultraviolet absorption spectra for *A*, undegraded filipin; *B*, filipin exposed to air at 70° for forty-eight hours; and *C*, filipin under nitrogen exposed to the same conditions. All are at 10 mcg./ml. concentration in absolute methanol.

filipin was investigated. In this kinetic study a spectrophotometric assay was used, and the absorbance at 355  $m\mu$  was statistically correlated with biological activity.

## EXPERIMENTAL

**Correlation of Spectrophotometric Data with Biological Activity.**—The calculated concentration<sup>2</sup> of thermally degraded filipin was statistically correlated with the *S. pastorianus* assay procedure of Bureh and Sokolski (6).<sup>3</sup> The spectrophotometric data used for this correlation were those obtained in the degradation rate study at 70°.

The statistics of correlation are represented by the plot in Fig. 2. The biological assay value (*P*) is given in terms of micrograms of filipin activity per milligram of sample as compared with a standard. The concentration (*C<sub>f</sub>*) of filipin as calculated from the observed absorbance at 355  $m\mu$  is defined as the fraction of the total material that is undegraded filipin.

The regression equation is  $P$  (mcg./mg.) =  $mC_f + b$  where *m* and *b* are slope and intercept, respectively. The actual equation is  $P = 967C_f + 36$ . The 95% confidence limits of the slope and intercept are  $\pm 148$  and  $\pm 104$ , respectively. A slope of 1,000 mcg./mg., which signifies a 1:1 ratio between the variables, and an intercept of zero can readily be accepted from these estimates of error. Thus it can be concluded that excellent correlation exists between spectrophotometric and biological assays. If the variation about the regression is assumed to be due to error in the bioassay and not a

function of the magnitude of the assay, the standard deviation of the *S. pastorianus* assay may be estimated as  $\pm 57$  mcg./mg. and the 95% confidence limits as  $\pm 126$  mcg./mg.

**Degradation Rate Studies.**—One-milliliter aliquots of a 5 mg./ml. methanolic solution of filipin were pipetted into 10-ml. amber vials and the solvent removed by vacuum distillation. The unsealed vials were then placed in covered 1-pound amber ointment jars and immersed in constant temperature baths at  $37 \pm 3$ ,  $50.0 \pm 0.5$ ,  $60.5 \pm 0.5$ , and  $70.0 \pm 0.5^\circ$ . The ointment jars were used (rather than just sealing the small vials) to provide a relatively large air space, thus keeping the oxygen concentration essentially constant. It was necessary to seal the system in some way because the samples were kept in a lighted room, and it was desired to keep the reaction nonphotolytic. Samples were periodically removed from the baths, diluted with absolute methanol to 10 mcg./ml., and assayed at 355  $m\mu$  using a Beckman model DU spectrophotometer.

In a preliminary experiment designed to study the stability of crystalline filipin in the absence of air, the same procedure was followed as for the air samples except that each vial was sealed with a rubber stopper and aluminum cap, then evacuated and filled with nitrogen by piercing the stopper with a hypodermic needle and alternately applying suction and nitrogen under pressure. In this manner the sealed vials were flushed with nitrogen three times and stored at 70°.

## RESULTS AND DISCUSSION

Curve A in Fig. 1 represents the spectrum of undegraded filipin, curve B the spectrum of filipin exposed to air at 70° for forty-eight hours, and curve C the spectrum of filipin exposed to nitrogen at 70° for forty-eight hours. It is readily apparent that the drug is much more stable in the absence of air.

In the aerobic degradation study, it was observed that the absorbance at 355  $m\mu$  did not go to zero in the expected length of time. Instead, it reached an asymptotic value which was different for each temperature (Fig. 3). When this value was reached, the absorbance declined at a rate much slower than the original.

The asymptotic values for the absorbances at the various temperatures are as follows:

Temperature °C.	Asymptotic Value, $A_{t_{\infty}}$
70.0	0.07
60.5	0.10
50.0	0.20
37.0	0.27

The above data indicated that the first degradation step(s) resulted in a product(s) which absorbed some light at 355  $m\mu$ ; this material then degraded further to give a nonabsorbing product(s). The fact that the asymptotic value was different for each temperature (it was practically zero at 70°) indicated that different degradation products, each having a different absorptivity, were formed at the various reaction temperatures. This agrees with findings on fumagillin reported by Garrett and Eble (4).

<sup>2</sup> The concentration of filipin was calculated from the observed absorbance (*b*) at 355  $m\mu$  in absolute methanol at 0.01 mg./ml. concentration. Theory and calculations are given under Results and Discussion.

<sup>3</sup> The authors are indebted to M. Bureh and co-workers of the Microbiology Department of The Upjohn Co. for the biological assays.

On the basis of the above postulates it became necessary to calculate the actual concentration of filipin at any time ( $t$ ). This would eliminate most of the error in the observed absorbance due to the contribution of the degradation products(s).

Let  $A_f$  = absorbance of filipin in methanolic solution (using a 1-cm. cell);  $a_f$  = absorptivity of filipin in methanol;  $C_f$  = concentration of filipin in the crystal lattice of the solid (defined as the fraction of total solids that is undegraded filipin); and  $v$  = volume of methanol in which  $C_f$  is dissolved to give absorbance  $A_f$ .

Then

$$A_f = a_f C_f / v \quad (\text{Eq. 1})$$

Similarly

$$A_x = a_x C_x / v \quad (\text{Eq. 2})$$

where  $x$  is the degradation product ( $v$  is constant throughout the calculations).

At any time ( $t$ ) let

$$C_f + C_x = 1 \quad (\text{Eq. 3})$$

and

$$A_t = A_f + A_x \quad (\text{Eq. 4})$$

where  $A_t$  is the observed absorbance at any time ( $t$ ).

Then

$$A_t = a_f C_f / v + a_x C_x / v \quad (\text{Eq. 5})$$

$$v A_t = a_f C_f + a_x (1 - C_f) \quad (\text{Eq. 6})$$

$$v A_t = a_f C_f + a_x - a_x C_f \quad (\text{Eq. 7})$$

$$v A_t - a_x = C_f (a_f - a_x) \quad (\text{Eq. 8})$$

$$C_f = (v A_t - a_x) / a_f - a_x \quad (\text{Eq. 9})$$

Equation 1 reduces to

$$a_f = v A_{t_0} \quad (\text{Eq. 10})$$

when  $t = 0$ , since then  $C_f = 1$ ,  $C_x = 0$ ,  $A_x = 0$ , and  $A_t = A_f$ . Similarly, Eq. 2 reduces to

$$a_x = v A_{t_\infty} \quad (\text{Eq. 11})$$

when  $t = \infty$ , since then  $C_f = 0$ ,  $C_x = 1$ ,  $A_f = 0$ , and  $A_t = A_x$ . The term  $t_\infty$  is defined as the time when filipin is completely degraded to  $x$  and  $x$  has not yet started to degrade. It follows that

$$C_f = \frac{v A_t - v A_{t_\infty}}{v A_{t_0} - v A_{t_\infty}} \quad (\text{Eq. 12})$$

and

$$C_f = \frac{A_t - A_{t_\infty}}{A_{t_0} - A_{t_\infty}} \quad (\text{Eq. 13})$$

Three assumptions are made here: (a) that filipin and its degradation products follow the Beer-Lambert Law; (b) that filipin is completely degraded to  $x$ , and (c) that the rate of degradation of  $x$  is insignificant compared with that of filipin. The first assumption is supported by the results of the bio-spectrophotometric correlation study.

The second introduces some error, since the aerobic degradation of a polyene may yield several products, each at a different stage of oxidation. However, for purposes of calculation it is conveni-

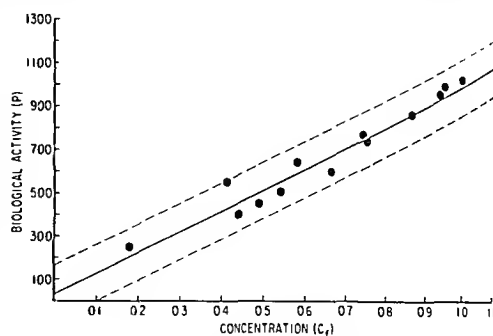


Fig. 2.—Plot of biological activity ( $P$ ) in mcg./ml. vs. calculated concentration ( $C_f$ ) of filipin as determined from U. V. assay at 355  $m\mu$ . The solid line is of best statistical fit and the dashed lines are 95% confidence limits of a predicted  $P$  from a specific  $C_f$ .

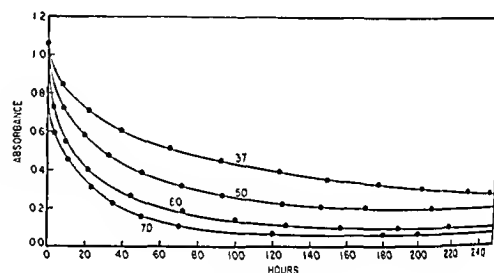


Fig. 3.—Plot showing the absorbances reaching asymptotic values, different for each temperature, as the reaction nears completion.

ent and reasonable to take  $a_x$  as the average absorptivity of all the degradation products whose concentration in the crystal lattice is  $C_x$ . This assumption is supported by the fact that equations resulting from it give the best fit to the data.

The third assumption is supported by the behavior of the absorbance values during the kinetic studies and by the fact that the deviations from the straight line plot (Fig. 4) do not appreciably increase as the reaction proceeds.

When the data were plotted, it was evident that the reaction was not zero or first order. Plots of the concentration of filipin and log of the concentration against time did not yield straight lines. Plotting the reciprocal of the concentration vs. time (Fig. 4) resulted in a straight line, indicating that the degradation was most probably second order with respect to filipin. The mechanism of the reaction is probably very similar to that proposed for the aerobic degradation of fumagillin by Garrett and Eble (4).

The reaction rate constants at the various temperatures, calculated from the slopes of the corresponding lines in Fig. 4, are as follows:

Temperature, °C.	Reaction Rate Constants ( $k$ ), hr. <sup>-1</sup>
70.0	0.178
60.5	0.126
50.0	0.081
37.0	0.042

An Arrhenius plot of the log of the reaction rate constants obtained in Fig. 4 against the reciprocals

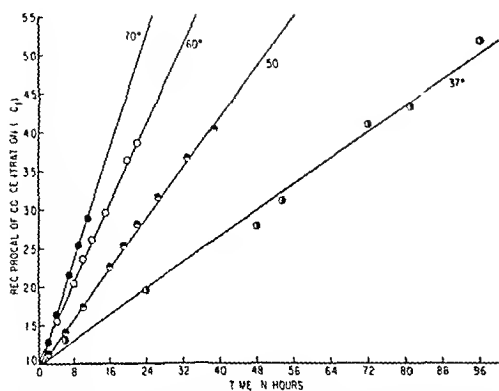


Fig 4—Plot illustrating the second order thermal degradation of crystalline filipin in the presence of air. The reciprocal of  $C_1$  is plotted against time in hours for several temperatures

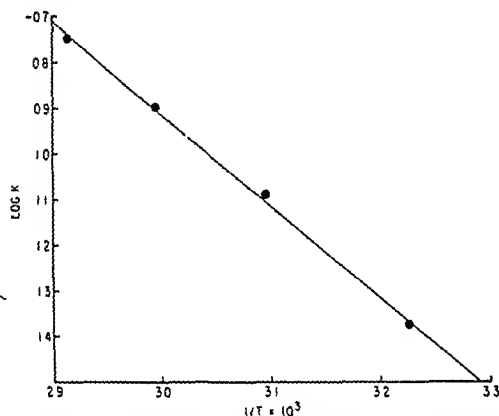


Fig 5—Arrhenius plot of the logarithm of the rate constant ( $k$ ) for the thermal oxidation of filipin against the reciprocal of the absolute temperature ( $T$ )

of the absolute temperatures is shown in Fig 5. The equation of best fit is

$$\log k = -2028/T + 5.17$$

The standard deviation of a predicted  $\log k$  at a given temperature is 0.014 and that of the slope is 63. The apparent heat of activation is 9.3 Kcal/mole. This heat of activation is very close to that for the aerobic degradation of fumagillin (4).

The half lives of filipin at 30, 25, and 4° were estimated to be thirty-three, forty-three and one hundred and forty-one hours, respectively<sup>4</sup>. At

<sup>4</sup> The half lives were calculated from the equation  $t_{1/2} = 1/kC_0$  where  $C_0$  is the initial concentration of filipin in the solid material. It can be seen that  $t_{1/2}$  is a function of initial concentration and that half lives in a second order reaction have little meaning unless initial concentration is specified. However, in this experiment it is assumed that the starting material was 100% filipin,  $C_0$  can then be normalized to 1 and the equation reduces to  $t_{1/2} = 1/k$ .

first glance, these data indicate that storage of the drug would be a major problem.

However, the environment to which the filipin was exposed in this study was much more severe than that which would exist under normal storage conditions. Removal of the methanol by vacuum distillation left the crystalline filipin with a much larger amount of exposed surface area than would be normally present. Furthermore, the oxygen supply was essentially unlimited, whereas a bottle nearly filled with drug and kept tightly closed for long periods of time (normal storage conditions) would contain a limited amount of oxygen.

These postulations are supported by results of studies on crystalline filipin reported by Whitfield (7) who found a 25% loss of potency at 25° and 13% loss at 4° after one year's storage in a closed amber bottle.

Preliminary experiments indicate that filipin is 50 to 100 times more stable in the absence than in the presence of air (see Fig 1). The half life of filipin in air at 70° is about six hours. The apparent half life of filipin under nitrogen at 70° is in excess of seven hundred and twenty hours. Results from the nitrogen experiments are not accurate due to leakage of air into some of the sealed vials, and the experiments will have to be repeated. However, air leakage would cause the apparent half life under nitrogen to be shorter than it actually is, therefore the true half life is probably longer than the one determined in these experiments.

## SUMMARY

1 Spectrophotometric absorbances at 355  $\mu$  give good indication of the progress of the aerobic degradation of crystalline filipin. Absorbances have been statistically correlated with biological assays.

2 Crystalline filipin is rapidly destroyed by heat in the presence of air. The reaction is oxidative and appears to be second order.

3 The mechanism of the reaction is probably similar to the one responsible for the aerobic degradation of fumagillin.

4 Preliminary experiments indicate that filipin is at least 50 to 100 times more stable under nitrogen than it is in air.

5 The empirical formula for filipin is now given as  $C_{27}H_{54}O_{10}$ .

## REFERENCES

- (1) Whitfield G B, Brock T D, Ammann A, Gottlieb D and Carter H E, *J Am Chem Soc* 77, 4799 (1955)
- (2) Garrett E R, and Eble T E, *THIS JOURNAL* 43, 385 (1954)
- (3) *Ibid*, 43, 536 (1954)
- (4) Garrett E R, *ibid* 43, 539 (1954)
- (5) Hughes H K, *Anal Chem*, 24, 1349 (1952)
- (6) Burch M R, The Upjohn Co., private communication
- (7) Whitfield G B, The Upjohn Co., private communication



# The Complexing Tendencies of Cyanocobalamin With Inorganic Compounds\*

## Heteromolybdates and Heavy Metal Chlorides

By RUTH N. HAVEMEYER† and TAKERU HIGUCHI

The interactions of vitamin B<sub>12</sub> with numerous inorganic compounds have been investigated. Nine of the compounds studied yielded slightly soluble adducts with cyanocobalamin. These were phosphotungstic and phosphomolybdic acid, the sodium, nickel, and manganese salts of phospho-12-molybdic acid, and the chlorides of gold, platinum, and palladium. Several of the reagents caused a change in the absorption spectrum of the vitamin in the system. This effect was found to be a function of the concentration of the reagent, but independent of hydrogen ion concentration.

THE ROLE OF COMPLEX FORMATION of cyanocobalamin (vitamin B<sub>12</sub>) as affects its absorption in the gastrointestinal tract has received much attention. There have been reports of the apparent interaction tendencies of the vitamin with mucoproteins, sugar alcohols, peptides, and other organic compounds (1-4).

In earlier work from these laboratories, studies were carried out on the association tendencies of vitamin B<sub>12</sub> with several classes of organic compounds. Szulcowski and Higuchi (5) studied the complex formation with sugars, sugar alcohols, and phenolic compounds. Mouri and Higuchi (6) interacted the vitamin with various carboxylic acids, amines, amides, and diols. The investigation reported at the present time is concerned with the binding of the vitamin with heteropoly acids and their salts, together with its interactions with some metal chlorides.

Studies of this nature were considered of pharmaceutical interest from two standpoints. It was hoped firstly that they may provide some insight into the influence of various additives upon the rate and efficiency of absorption of the vitamin when taken orally. Secondly, it was thought that some dosage form of the depot type might be based on the discovery of some extremely insoluble complexes formed from cyanocobalamin. It would be pharmacologically desirable to have available dosage forms of vitamin B<sub>12</sub> which would permit its slow and controlled release into the system. One possible way to realize this might be to form slowly soluble molecular addition compounds, or complexes, suitable for parenteral use.

Such interactions may reasonably be expected.

The vitamin B<sub>12</sub> molecule (Fig. 1) includes several groups which might conceivably act as complexing sites; these are the amide function, the orthophosphate moiety, the cyano-cobalamin system, and the nucleotide portion of the molecule. Interactions of some of these functional types are well-known. For example, orthophosphates complex strongly with ferric ion (7), and simple amides such as acetamide and benzamide form crystalline addition compounds which can be isolated from aqueous solution, with gold and platinum chlorides (8, 9). Many inorganic compounds function as protein precipitants (10, 11), and some of these may well be expected to act similarly upon the cyanocobalamin structure.

Since poorly soluble or slowly soluble adducts were desired, some substances of high molecular weight were employed as the complexing agents. Other compounds, such as gold chloride, were

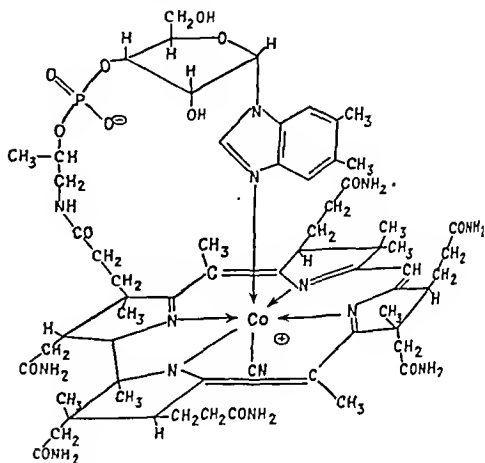


Fig. 1.—The structure of cyanocobalamin. Reprinted by permission, from p. 19 of "Vitamin B-12 and Intrinsic Factor," H. C. Heinrich, editor.

\* Received August 21, 1959, from the School of Pharmacy, University of Wisconsin, Madison.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: Squibb Institute for Medical Research, New Brunswick, N. J.

chosen on the basis of known interactions with pertinent functional groups. Still others were selected for study in an attempt to separate out specific ion effects. In addition, such studies may yield some information as to the sites of interaction.

## EXPERIMENTAL

**Reagents.**—*Heteromolybdates.*—Sodium-phospho-12-molybdate, sodium-silico-12-molybdate, sodium-2-phospho-18-molybdate, Climax Molybdenum Co.; phosphotungstic acid, Eastman Kodak; phosphomolybdic acid, Merck and Co.; manganese-phospho-12-molybdate, nickel-phospho-12-molybdate.

The first five compounds were recrystallized from hot water. The last two were not commercially available and were synthesized by the method of Arnfeld, as follows (12): Freshly precipitated nickelous (or manganous) phosphate was suspended in hot water. To this was added, in small portions, the stoichiometric quantity of molybdenum trioxide which had been recently heated to glowing in a porcelain crucible. The suspension was heated gently and small quantities of water were added until all of the solids dissolved. This solution was concentrated over sulfuric acid and yielded greenish-yellow crystals. The first crystals to separate, those of the phospho-12-molybdic acid, were filtered off in order to separate them from the salt of the 9-acid, which are the last to separate. An aqueous solution of the 12-acid salt, when treated with ammonium or potassium chloride, yields a precipitate. A solution of the 9-acid salt shows no reaction with either of these reagents.

**Chlorides.**—Aluminum, ammonium, barium, cadmium, cobaltous, cupric, ferric, hexamino-cobalt, lithium, lead, manganous, mercuric, nickelous, sodium, zinc, auric, platinum, palladous, and potassium gold chlorides.

All but the last four compounds were recrystallized from hot water. These four chlorides were used as supplied by the manufacturers; they were not recrystallized because of their hygroscopicity and because of the small quantities available.

**Procedure.**—The method of study was phase solubility analysis, using a water bath thermostatted at  $25 \pm 0.05^\circ$ . The method and the apparatus have been described previously (13, 14).

The crystalline vitamin  $B_{12}$  was weighed into 2-cc. vials and sufficient water was added to give a concentration of 20 mg.  $B_{12}$ /Gm. water. Sufficient reagent was then added to give the desired concentrations. After a twenty-four-hour equilibrium period, the supernatant liquid was withdrawn through medium-porosity sintered-glass disks. These aliquots were placed in tared 10-ml. volumetric flasks, and the weights of the aliquots were determined. The diluted samples were analyzed for  $B_{12}$  content by measuring their absorbance at 550  $m\mu$ , using the Cary recording spectrophotometer model 11MS.

All solutions containing  $B_{12}$ , in free or combined form, were saved for recovery of the vitamin. The pooled solutions were first lyophilized, and the dry powder obtained was redissolved to give a concentrated solution. This concentrate was then treated

by the method of Bernhauer and Friedrich (15) to obtain a purified aqueous solution, and the crystalline vitamin was finally precipitated by addition of 90% acetone. The acetone used was first purified by refluxing it for several hours with potassium permanganate and potassium carbonate, and was then distilled. The reclaimed vitamin was checked for purity spectrophotometrically.

**Sample Calculation to Determine the  $B_{12}$  Content of the Diluted Aliquots.**—(a) (Absorbance) (volume of final soln./absorptivity) = mg.  $B_{12}$ /10 ml., (b) (mg.  $B_{12}$ /10 ml.) (1/weight of aliquot) = mg.  $B_{12}$ /Gm. (c) (mg.  $B_{12}$ /Gm.)  $\div$  (molecular weight of  $B_{12}$ ) =  $10^{-6}$  mole  $B_{12}$ /Gm.

## RESULTS

**Heteromolybdates.**—Figure 2 shows the curves for those interactions which resulted in only an increase in the solubility of cyanocobalamin; Figs. 3 and 4 give the complexing curves for those compounds which interacted with  $B_{12}$  to give an insoluble form.

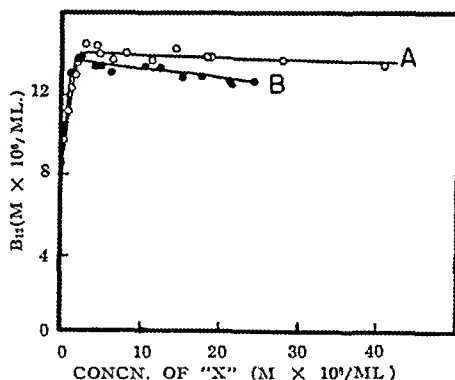


Fig. 2.—A, sodium-silico-12-molybdate; B, sodium-2-phospho-18-molybdate.

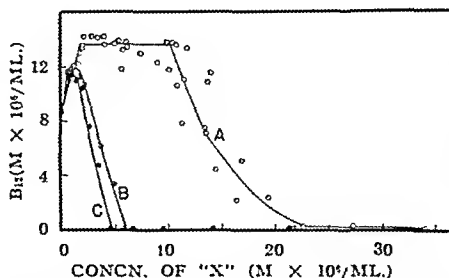


Fig. 3.—A, sodium-phospho-12-molybdate; B, nickel-phospho-12-molybdate; C, manganese-phospho-12-molybdate.

The scatter observed may be the cumulative result of the experimental errors, the principal one of which is probably the weighing error. It is not the result of insufficient equilibration, since the same effect was observed when the equilibrium time was extended to forty-eight hours.

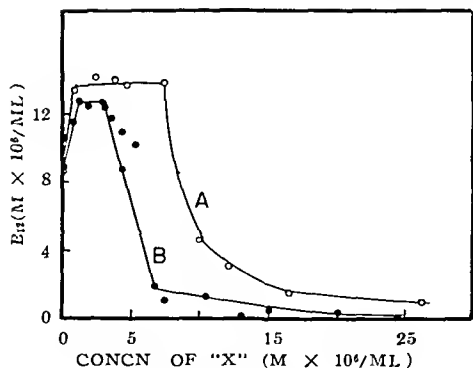


Fig 4—A, phosphotungstic acid; B, phosphomolybdic acid

The data for the compounds which gave insoluble reaction products are given in Table I. The approximate stoichiometries of the solid complexes were calculated in three ways: (a) from the initial slope of the descending portion of the curve; (b) from the amount of complexing agent necessary to remove all of the vitamin from the system, determined by extrapolating the downcurve of the plots to the  $x$ -axis. The value, total  $B_{12}$ /total agent, is the ratio of the concentration of the vitamin to this value; and (c) by spectral analysis, accomplished by calculating the concentration of  $B_{12}$  from the absorbance of a solution of 1 mg of the solid in 25 ml. of water.

Of the three methods, the spectral analysis of the solid is probably the most reliable. Since the other two are based upon calculations from the phase diagrams they have rather large inherent errors in them. The downslopes, for example, are only best-fitting lines by visual (not mathematical) analysis, and in at least one of the curves there is considerable scatter. Also, because of the steepness of the downslopes, it is often difficult to obtain precise numerical values therefrom.

**Chlorides.**—Of the nineteen chlorides studied, platonic, auric, palladous, and potassium gold chlorides gave insoluble reaction products with cyanocobalamin (Figs 5 and 6, Table II). Mercuric chloride is the only one which did not cause an initial increase in the solubility of the vitamin (Fig 7).

Gold, potassium gold, and palladium chlorides caused a shift in the spectrum of  $B_{12}$  in the supernatant liquid. In the case of the gold salts, the two maxima at 550  $m\mu$  and 520  $m\mu$  blended into one

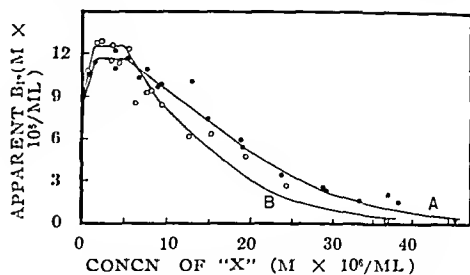


Fig 5—A, potassium gold chloride; B, palladium chloride.

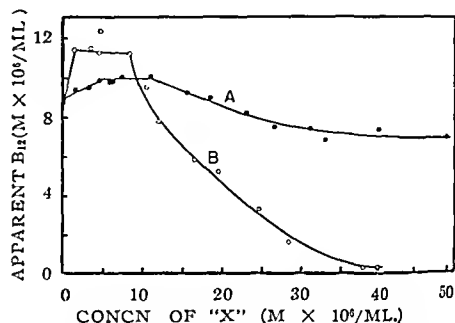


Fig. 6—A, platinum chloride; B, gold chloride

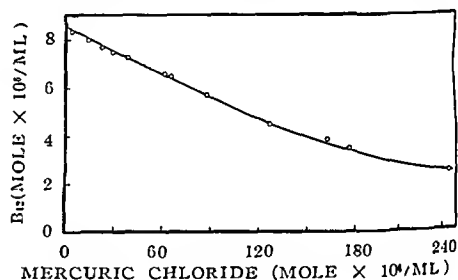


Figure 7.

maximum at about 525  $m\mu$ . This change is a function of concentration of the salt added but is independent of hydrogen ion concentration, as shown by a pH-dependence study using HCl.

With palladous chloride, also, the spectral shift was dependent upon the concentration of the chloride added. However, the spectral change observed in this system was more complicated, several changes in spectrum being noted with changing palladium concentration.

TABLE I—THE HETEROMOLYBDATES

Reagent	Downslope	Approximate Molar Ratio ( $B_{12}$ :Agent) of Solid Complex	
		Total $B_{12}$	Spectral Analysis
		Total Agent	
Na-phospho-12-molybdate	10:3 (?)	1:1	1:1
Mn-phospho-12-molybdate	7:2	7:2	6:1
Ni-phospho-12-molybdate	3:1	5:2	5:2
Phosphomolybdic acid	2.7:1	2:1	1.7:1
Phosphotungstic acid	6:1 (?)	8:5 (?)	4:1

## DISCUSSION

**Heteromolybdates.**—The heteropoly anions are composed of molybdenum oxide groups which surround a central atom such as potassium, tungsten, or silicon. For example, in the phospho-12-molybdates, such as  $Na_3(PMo_{12}O_{40})$ , there is a  $PO_4$  tetrahedron surrounded by twelve  $MoO_6$  octahedra (Fig 8). These twelve octahedra are joined together by the sharing of oxygen atoms at the corners. In the hetero-12-molybdates, there are twelve molybdenum atoms to one central atom; in the hetero-2,18-molybdates, there are eighteen molybdenum atoms to two central atoms. The structures of the two complex acids used are similar; each consists of a

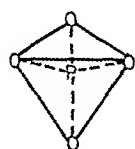
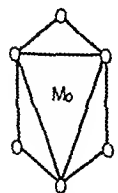
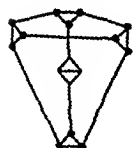
TABLE II.—THE TRANSITION METAL CHLORIDES

Reagent	% Change in $B_{12}$ Solubility <sup>a</sup>	Approx Stoichiometry of Solid Complex ( $B_{12}$ : Agent)	Method of Calculation	Spectral Analysis of Precipitate	Upper limit of Reagent Concn (10 <sup>-6</sup> Mole/ml)
HAuCl <sub>4</sub>	-100	3:2	Total $B_{12}$ : Total Agent	1:1	incompletely soluble <sup>b</sup>
H <sub>2</sub> PtCl <sub>6</sub>	-16	1:7		2:3	..
KAuCl <sub>4</sub>	-100	2:5		1:2	incompletely soluble
PdCl <sub>2</sub>	-100	1:1		4:5	insoluble <sup>c</sup>
HgCl <sub>2</sub>	-71 <sup>d</sup>				..
PbCl <sub>2</sub>	+0.6				30
NaCl	+4				5480
LiCl	-9				90
CoCl <sub>2</sub> 6H <sub>2</sub> O	+15				44
NiCl <sub>2</sub> 6H <sub>2</sub> O	+15				49
CdCl <sub>2</sub>	+15				114
MnCl <sub>2</sub> 4H <sub>2</sub> O	+18				50
CuCl <sub>2</sub> 2H <sub>2</sub> O	+20				74
BaCl <sub>2</sub>	+23				115
ZnCl <sub>2</sub>	+30				110
NH <sub>4</sub> Cl	+30				656
AlCl <sub>3</sub>	+30				46
[Co(NH <sub>3</sub> ) <sub>6</sub> ]Cl <sub>3</sub>	+53				173
FeCl <sub>3</sub> 6H <sub>2</sub> O	+65				58

<sup>a</sup> + Indicates increase in solubility and - indicates decrease in solubility, calculated at reagent concentration in column 6. <sup>b</sup> One milligram incompletely soluble in 25 ml of water. <sup>c</sup> One milligram insoluble in 25 ml water. <sup>d</sup> See Fig 7.

$PO_4$  tetrahedron surrounded by six  $XO_6$  octahedra, where X is molybdenum or tungsten. These compounds are generally highly hydrated and may contain as many as forty molecules of water. Some of these water molecules are intimately bound in the crystal lattices and others are apparently zeolytic, i.e., they are lost on heating because they are not part of the crystal structure (16, 17).

The molecular weights of the heteromolybdates range from 2,000 to 4,000. Because of the complexity of these anions, there is still much confusion as to their structures and bond types. Another complication is that in solution there is an equilibrium between several of the anionic species.

THE  $PO_4$  TETRAHEDRONTHE  $Mo_6O_6$  OCTAHEDRON

THE PHOSPHO-12-MOLYBDATE MOLECULE  
EACH  $\bullet$  REPRESENTS ONE  $Mo_6O_6$  OCTAHEDRON  
HENCE, THERE ARE  
12  $Mo_6O_6$  GROUPS:  $PO_4$  GROUP

Figure 8.

From the complexing data, it would appear that for the several compounds studied, the ratio of 12 Mo: 1 P in the structure of the heteromolybdate is required to obtain an insoluble or slowly soluble adduct with the vitamin.

**Chlorides.**—The first chlorides studied were those of gold and platinum, since it was known that they react with acetamide groups (8, 9). The results of these interactions with vitamin  $B_{12}$  led to the investigation of other chlorides.

The spectrum change observed as a result of the interactions of the vitamin with gold and potassium gold chlorides is similar to that reported by Kaczka, *et al.*, on work done with various  $B_{12}$  analogs, in which the cyanide group coordinated to the cobalt in the  $B_{12}$  molecule was replaced with hydroxide, chloride, bromide, sulfide, sulfate, nitro, and cyanate groups (18). Hence, it is possible that cyanocobalamin is no longer present in solution. Possible causes of such spectral shifts may be replacement of the cyanide by other anions, interaction of the cation with the molecule in such a way as to disturb the conjugated bond system which is responsible for the characteristic absorption spectrum of vitamin  $B_{12}$ , or oxidation-reduction reactions due to the presence of the auric ion. The shifted peak obtained in this study, 525  $m\mu$ , could conceivably correspond to that of cyanatocobalamin (18).

A photometric study was undertaken in which a saturated solution of gold chloride or palladium chloride was added dropwise to a rapidly stirred vitamin  $B_{12}$  solution. The titration flask was connected to a Corex cell (19), and the spectrum of the resultant solution was recorded after each addition of the metal chloride. In each case, the reaction which resulted in the changing spectrum was found to be time dependent.

The spectral changes observed in the palladium chloride-cyanocobalamin system seem to indicate that there are several complex species forming.

We were unable to determine isosbestic points in those samples in which the agent was present in concentrations low enough to form soluble species, and it may be that at all reasonable concentrations of palladium chloride there are more than two species in equilibrium. Since the palladium salt used, palladium chloride, is not palladium in its highest oxidation state, a more complex oxidation-reduction reaction may be occurring. It may be for this reason that the spectral shift is not constant.

The infrared spectra of several of the insoluble reaction products were inconclusive as regards the nature of the reactions taking place.

The decrease in solubility observed with mercuric chloride and vitamin B<sub>12</sub> may be the result of a salting out effect or a medium effect upon the absorption spectrum. The latter is perhaps a more feasible explanation, since the concentrations of mercuric chloride used were very low. It seems reasonable to expect that if there were complex formation between these compounds, then an initial plateau region would result until all of the solid excess B<sub>12</sub> in the system had reacted.

The majority of the metal chlorides employed in this study resulted in an increase in the solubility of the cyanocobalamin (Table II), but without a subsequent decrease. These compounds may be of value as additives to aid in the increased absorption of vitamin B<sub>12</sub> preparations, or they may prove to be useful in the stabilization of the vitamin in solution.

## REFERENCES

- (1) Grasbeck, R., *Acta Med Scand*, Suppl 154, 314(1957)
- (2) Greenberg, S. M., Herndon, J. F., Rice, E. G., Paley, E. T., Gulesich, J. J., and Van Loon, E. J., *Nature*, 140(1957)
- (3) Heathcote, J. G., and Mooney, F. S., *Lancet*, 1, (1958)
- (4) Heinrich, H. C., "Vitamin B<sub>12</sub> und Intrinsic Factor," Europäisches Symposium, Hamburg 1956," Ferdinand Verlag, Stuttgart, Germany, 1957
- (5) Szulcowski, D., M.S. Thesis, University of Wisconsin, 1957
- (6) Moury, Y., and Higuchi, T., Unpublished data
- (7) Van Wazer, J. R., and Callis, C. F., *Chem Revs*, 101(1958)
- (8) Fricke, R., and Ruschhaupt, F., *Z anorg u all Chem*, 146, 141(1925)
- (9) Barkowski, V. F., and Kulberg, L. M., *Akad N S S S R*, 28, 235(1954), *Chem Abstr*, 50, 801(1956)
- (10) Goodman, L. S., and Gilman, A., "The Pharmacological Basis of Therapeutics," 2nd Ed., MacMillan Co., New York, N. Y., 1956, p. 1102 ff
- (11) Wu, H., *J Biol Chem*, 43, 189(1920)
- (12) Arnfeld, A., *Bertrage zur Kenntnis der Phosphomolybdate* Dissert., Berlin, Germany, 1898
- (13) Zuck, D., and Higuchi, T., *THIS JOURNAL*, 42, (1953)
- (14) Higuchi, T., Gupta, M., and Busse, L., *ibid*, 157(1953)
- (15) Bernauer, K., and Friedrich, W., *British pat* 7, 982, July 17, 1957
- (16) Pauling, L., in "Molybdenum Compounds Their Chemistry and Technology," by D. Killeffer and A. L. Interscience Publishers, Inc., New York, N. Y., 19 Chapter 9
- (17) Climax Molybdenum Co., Bulletin Cdh 12, October 1956
- (18) Kaczka, E. A., Wolf, D. E., Kuehl, F. A., Jr., and Folkers, K., *J Am Chem Soc*, 73, 3569(1951)
- (19) Rehm, C., Bodin, J. I., Connors, K. A., and Higuchi, T., *Anal Chem*, 31, 483(1959)

## Antioxidants. The Microdetermination of Hydroquinone\*

By MOHAMED Z. BARAKAT, SAAD K. SHEHAB, and ABDEL MAKSOUD ABDALL.

A new titrimetric method for the microdetermination of hydroquinone is described. This method is recommended for the assay of fats and oils, photographic developers, and ether anesthetics. The assay is carried out within limits of 0.5 to 2 mg. of hydroquinone. The procedure is quite simple and rapid but yet shows relatively high accuracy over the suggested range. The experimental error does not exceed  $\pm 2$  per cent.

HYDROQUINONE is an effective antioxidant for fats and oils (1-5). In tropical districts, hydroquinone is used as a preservative for ether anesthetics (6). Furthermore, hydroquinone is a prominent constituent in photographic developers

(7). Hence, our interest in the microdetermination of hydroquinone.

Previously, various methods have been reported (8-12) for the determination of hydroquinone. Of these, the titrimetric methods are used extensively because of their rapidity and simplicity. So far as we are aware, certain de-

\* Received July 22, 1959, from the Faculty of Veterinary Medicine, Cairo University, Giza, Cairo, Egypt

fects are involved in the previously known volumetric methods (13-16). Moreover, the reported experimental error (17) is outstanding, being  $\pm 20$  per cent.

The present investigation deals with a new easy, rapid, and accurate titrimetric method for the microdetermination of hydroquinone

### EXPERIMENTAL

**Equipment.**—A microburet of 5-ml capacity, graduated in hundredths of a milliliter, graduated pipets of 1-, 2-, and 5-ml capacity, Erlenmeyer flasks of 25-, 50-, 100-, and 250-ml capacity

**Reagents.**—Aqueous potassium iodide solution, 4% w/v, aqueous starch solution, 1% w/v, a saturated aqueous sodium bicarbonate solution; aqueous N-bromosuccinimide solution, 0.1% w/v, which may be serially diluted 10 times as required

#### Validity of Reaction for Quantitative Estimation.

—The authors decided to verify the action of N-bromosuccinimide on hydroquinone (18) from a quantitative point of view. Practically it was found that one molecule of hydroquinone was oxidized by one molecule of N-bromosuccinimide. The reaction was also valid to estimate hydroquinone in concentrations as low as 55  $\gamma$  per 5 ml. of solution

**Procedure**—Into a 50-ml Erlenmeyer flask, a known volume of the unknown hydroquinone solution is introduced, i. e., 1 ml contains 1 mg of hydroquinone. Then an equal volume of saturated sodium bicarbonate solution, 5 ml of 4% potassium iodide solution, and 10 drops of starch solution as an indicator are added. The aqueous N-bromosuccinimide solution (0.1%) is introduced into the microburet and is allowed to run, drop by drop, into the hydroquinone solution, with continuous shaking. The end point is reached when the last drop of the N-bromosuccinimide solution added produces a permanent blue color in the hydroquinone solution (Table I)

A 0.1% hydroquinone solution was estimated simultaneously, with 0.01 N iodine (Table II)

### METHOD OF ASSAY

**Fats and Oils.**—For the estimation of hydroquinone in fats or oils, the following method is recommended. A known weight or volume of the fat (10 Gm) or oil (10 ml), respectively, containing 0.5 to 2 mg of hydroquinone, is dissolved in 150 ml of ether; in case of oils the pipet being washed twice with ether. Then the procedure for estimation of pure hydroquinone solutions is followed but the end point is violet

This method has been successfully applied to butter, lard, and cod liver oil. Hydroquinone (0.1 Gm) was dissolved in ether (100 ml) in a stoppered standard flask and 20 ml of this ethereal solution (containing 20 mg of hydroquinone) was added to 100 Gm of freshly melted butter or lard and to 100 ml of cod liver oil. The ether was evaporated and each sample was allowed to cool. Consequently, 10 Gm of butter or lard and 10 ml of cod liver oil should contain 2 mg of hydroquinone (Table III).

TABLE I.—ESTIMATION OF PURE HYDROQUINONE SOLUTIONS BY 0.1% NBS<sup>a</sup>

Hydroquinone content =  $V \times C \times 110/178$  (mg. or mcg) where  $V$  = volume of N-bromosuccinimide solution and  $C$  = concentration of N-bromosuccinimide solution either in mg or mcg

Hydroquinone Soln, %	Volume, ml	Content, mg	0.1% NBS Used, ml	Found, mg	Error, %
0.5	1	5	8.15	5.04	0.80
	2	10	16.20	10.01	0.10
0.4	1	4	6.45	3.99	0.25
	2	8	13.00	8.03	0.38
0.3	1	3	4.85	3.00	
	2	6	9.90	6.12	2.00
0.2	3	9	14.50	8.96	0.44
	1	2	3.20	1.98	1.00
	2	4	6.50	4.02	0.50
	3	6	9.70	5.99	0.17
	4	8	13.00	8.03	0.38
	5	10	16.20	10.01	0.10
0.1	10	10	16.00	9.89	1.10
	9	9	14.60	9.02	0.22
	8	8	13.00	8.03	0.38
	7	7	11.40	7.04	0.57
	6	6	9.90	6.12	2.00
	5	5	8.15	5.04	0.80
	4	4	6.55	4.05	1.25
	3	3	4.85	3.00	
	2	2	3.25	2.01	0.50
	1	1	1.60	0.99	1.00

<sup>a</sup> N-Bromosuccinimide solution

TABLE II.—COMPARATIVE ANALYSIS BY PROPOSED METHOD AND BY IODINE

0.1% Hydroquinone Soln, ml	Content, mg	Found by 0.1% NBS, mg	Error, %	Found by 0.01 N Iodine, mg	Error, %
10	10	9.98	0.20	10.01	0.10
8	8	8.03	0.38	7.98	0.25
6	6	6.05	0.83	6.05	0.83
4	4	3.96	1.00	4.13	3.25
2	2	2.04	2.00	2.09	4.50
1	1	1.01	1.00	1.045	4.50

TABLE III.—DETERMINATION OF HYDROQUINONE IN BUTTER, LARD, AND COD LIVER OIL

Sample	Weight of Fat or Volume of Oil, Gm or ml	Hydroquinone Content, mg	0.1% NBS Used, ml	Hydroquinone Found, mg	Error, %
Butter	10	2	3.20	1.98	1.00
Lard	10	2	3.18	1.97	1.50
Cod liver oil	10	2	3.30	2.04	2.00
	5	1	1.60	0.99	1.00
	2.5	0.5	0.80	0.49	2.00

**Photographic Developers.**—One example is sufficient to illustrate the validity of the assay method, e. g., the following solution:

Metal.	0.1 Gm.
Sodium sulfite	1.3 Gm.
Hydroquinone	0.3 Gm.
Sodium carbonate	2.6 Gm.
Potassium bromide	0.1 Gm.
Water ad...	100 ml.

**Method.**—To 2 ml of the developer add 20 ml of 10% barium chloride solution, shake well, and filter after ten minutes (away from sunlight) To the filtrate apply the same procedure for pure hydroquinone solutions Only the end point is violet

Developer, ml	Hydro- quinone Content, mg	0.1% NBS Used, ml	Hydro- quinone Found, mg	Error, %
2	6	9.90	6.12	2.00

**Ether Anesthetics.**—The same procedure for pure hydroquinone solutions is adopted The results of estimating 1% w/v of hydroquinone in ether and a serial dilution (10 times) of it are given in Table IV

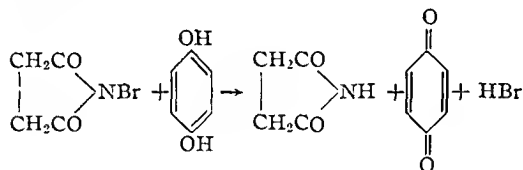
## RESULTS

**Interfering Substances.**—The only interfering substance that is also oxidized by N-bromosuccinimide before iodine is liberated from potassium iodide includes sulfite present in photographic developers but it is eliminated in the form of insoluble barium sulfite Metol has no influence on the titration process

**Experimental Error.**—From the results given in Tables I to IV, it has been deduced that the error of the proposed method does not exceed  $\pm 2\%$

## DISCUSSION

N-Bromosuccinimide in aqueous medium readily oxidizes an aqueous solution of hydroquinone to benzoquinone, while N-bromosuccinimide is irreversibly reduced to succinimide with the formation of hydrogen bromide The reaction proceeds quantitatively, in the presence of sodium bicarbonate, in equimolecular concentrations according to the equation



Sodium bicarbonate eliminates hydrogen bromide as soon as it is formed and consequently the reaction proceeds forward to completion The fact that hydroquinone is selectively oxidized by N-bromosuccinimide (19, 20) before iodine is liberated from potassium iodide, provides a reliable titrimetric method for the determination of hydroquinone

N-Bromosuccinimide is an oxidizing agent and thus can liberate iodine from potassium iodide in

TABLE IV—ESTIMATION OF HYDROQUINONE IN ETHER<sup>a</sup>

Volume of Ether, ml	Hydroquinone Content, mg	1% NBS Used, ml	Hydroquinone Found, mg	Error %
1	10	16.20	10.01	0.1
10	10	16.30	10.07	0.7
8	8	13.10	8.10	1.2
6	6	9.80	6.06	1.0

<sup>a</sup> Dilution, 10X, 1 mg per ml

aqueous medium, but it oxidizes hydroquinone preferentially Until all the hydroquinone present in the solution is oxidized, no iodine is liberated from potassium iodide The slightest excess of N-bromosuccinimide added, after all the hydroquinone content has been oxidized, will liberate iodine from potassium iodide, which is easily detected by the blue color developed with a few drops of starch solution added at the beginning of the titration process The end point is definitely blue in the case of pure solutions but violet in the case of fat oils, and photographic developers

The proposed method is applicable even to such low concentration as 55 mcg. of hydroquinone Only 0.01% N-bromosuccinimide is used in the titration process and a blank experiment is simultaneously carried out The blank reading (0.09 m of 0.01% NBS) is subtracted from the titration result before calculation is made

## REFERENCES

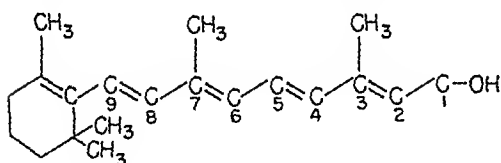
- (1) Hebbel, H. S., and Martini, G. R., *Anales quim. farm. Santiago, Chile*, 1944, 1, *Chem. Abstr.*, 39, 2417 (1945)
- (2) Bucher, D. L., *Fishery Market News*, 7, 17 (1945)
- (3) Nair, F. V., and Ramakrishnan, T. A., *Current Sci. India*, 13, 232 (1944)
- (4) Tauffel, K., and Arens, E., *Fette u. Seifen*, 51, 13 (1944), *Chem. Abstr.*, 42, 9203 (1948)
- (5) Sandell, E., *Stenssk. Farm. Tidskr.*, 54, 473, 501, 53 (1950)
- (6) Ghosh, B., and Bhattacharya, A., *J. Proc. Ind. Chemists India*, 18, 154 (1946)
- (7) Strauss, P., *Foto Kino Tech.*, 3, 30 (1949), *Chem. Abstr.*, 43, 8921 (1949)
- (8) Vaskevich, D. N., and Gol'dina, T. A., *Zhur. Prikl. Khim.*, (*J. Appl. Chem.*), 24, 1214 (1941), *Chem. Abstr.*, 41, 2964 (1952)
- (9) Belcher, R., and West, T. S., *Anal. Chim. Acta*, 599 (1951)
- (10) Exner, J., and Bobdaneky, M., *Chem. listy*, 49, 109 (1955), *Chem. Abstr.*, 49, 14578 (1955)
- (11) Caulfield, F. H., and Robinson, R. J., *Anal. Chem.*, 25, 982 (1953)
- (12) Singh, B., and Singh, A., *Anal. Chim. Acta*, 9, 2 (1953)
- (13) Kolthoff, I. M., *Rec. trav. chim.*, 43, 743 (1926)
- (14) Preiss, W., *Z. Untersuch. Lebensmittel.*, 67, 144 (1934)
- (15) Pevtsov, G. A., *Zavodskaya Lab.*, 7, 110 (1938)
- (16) Bielenberg, W., Goldhahn, H., and Zoff, A., *Org. Kohle*, 37, 496 (1941)
- (17) Fiserova, V. B., and Skramovsky, S., *Pracovi. Lékařství*, 4, 64 (1952), *Chem. Abstr.*, 49, 4072 (1955)
- (18) Barakat, M. Z., Abd El-Wahab, M. F., and El Sadr, M. M., *J. Am. Chem. Soc.*, 77, 1670 (1955)
- (19) Fieser, L. F., and Rajagopalan, S., *ibid.*, 71, 3933 (1949), 72, 5530 (1950)
- (20) Barakat, M. Z., Abd El-Wahab, M. F., and El Sadr, M. M., *Anal. Chem.*, 27, 536 (1955)

# Isomerization of Vitamin A in Aqueous Multivitamin Drop Preparations\*

By ROBERT W. LEHMAN, JOHN M. DIETERLE, WILLIAM T. FISHER,  
and STANLEY R. AMES

Seven samples of aqueous multivitamin drop formulations were prepared using three different samples of all-*trans*-vitamin A palmitate. These were assayed after three, six, nine, and twelve months' storage at 37°, and after nine, twelve, and fifteen months' storage at 25°. The rate of apparent deterioration of vitamin A was dependent on the assay method used, being least for the antimony trichloride blue-color assay, intermediate for the U. S. P. spectrophotometric assay, and greatest for the rat liver-storage bioassay. The vitamin A isomer composition was studied by reaction with maleic anhydride and by examination of infrared absorption spectra of purified vitamin A aldehydes made from the preparations. Results of this study indicate that during storage in the multivitamin drop preparations, all-*trans*-vitamin A isomerizes to a mixture containing not only all-*trans*- and 2-mono-*cis*- (neo-vitamin A), but also significant quantities of 6-mono-*cis*- and 2,6-di-*cis*-isomers, which have low biological activity.

VITAMIN A (see Fig 1) has five conjugated double bonds, some of which can exist in either the *trans*- or *cis*- configuration. In 1939, Pauling (1) postulated that two of these double bonds, 2—3 and 6—7, were "stereochemically effective" and could exist readily in either the *trans*- or *cis*-configuration, and that other isomers were unlikely because they would be sterically hindered. These isomers have been prepared by Robeson, *et al* (2), and their properties determined (2, 3, 4). Table I gives some of the analytical properties in terms of the "potencies" obtained by different assay procedures



all-*trans*                      6-mono-*cis*  
2-mono-*cis* (neo)              2,6-di-*cis*

Fig 1—Isomers of vitamin A

## EXPERIMENTAL

In our present studies we prepared seven aqueous multivitamin drop preparations. They were made with commercial, all-*trans*-vitamin A palmitate, contained Tween 80 as the dispersing agent, and were adjusted to pH 5.3. All contained about 6,000 U. S. P. units of vitamin A, 1 mg. of thiamine,

and 50 mg. of ascorbic acid in a "dose" of 0.6 cc. In addition, four of the preparations also contained 0.4 mg. riboflavin, 2 mg. calcium pantothenate, and 5 mg. nicotinamide per 0.6 cc. The preparations were divided into individual bottles and tightly capped under nitrogen for assay after storage for three, six, nine, and twelve months at 37°, and for nine, twelve, and fifteen months at room temperature (controlled at 25°).

The rate of apparent deterioration of vitamin A was measured by three different assay methods: the antimony trichloride blue-color procedure (4), the U. S. P. XV spectrophotometric procedure (5), and the rat slope ratio liver-storage biological assay (6).

The recovery data obtained for the preparations stored at 37° are summarized in Fig 2. Antimony trichloride blue color determinations and U. S. P. spectrophotometric assays were obtained on practically all of the preparations at all storage periods. Bioassays were obtained on several of the preparations. Similar data at 25° are given in Fig 3.

It is apparent that two different changes are taking place at the same time. The vitamin A is decomposing chemically, and this is the change of greatest magnitude. However, as decomposition takes place, there arises a systematic discrepancy between three different assay procedures. The apparent rate of decomposition is least by the antimony trichloride blue-color assay, it is intermediate by the U. S. P. XV assay, and it is greatest by the rat liver-storage bioassay.

One explanation for the divergence of the three assay procedures is that vitamin A isomerizes. Reference to Table I will illustrate how vitamin A isomers respond to the different assay methods. A mixture of the four isomers should be expected to have a lower "potency" by U. S. P. XV assay than by antimony trichloride blue color, since all four isomers give the same colorimetric value while three of the isomers have lowered U. S. P. values. Likewise, the biological potency of a mixture of four isomers should be lower than that obtained by either of the physicochemical assay procedures.

The per cent recoveries found by each assay procedure, as presented in Figs 2 and 3, are averaged

\* Received August 21, 1959 from the Laboratories of Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y.

Presented to the Scientific Section A Pitt A, Cincinnati meeting, August 1959.

The expert technical assistance of H. A. Risley and W. J. Swanson of the D. P. I. Biochemical Research Department, M. H. Stern and W. P. Blum of the D. P. I. Organic Research Department, and G. H. Wait of the laboratory greatly assisted.



TABLE I—PROPERTIES OF GEOMETRIC ISOMERS OF VITAMIN A PALMITATE<sup>a</sup>

Isomer	Antimony Trichloride Blue-Color "Potency," u/Gm	U S P XV "Potency," u/Gm	Relative U S P XV "Potency," % of Blue-Color	Biological Potency, u/Gm	Relative Biopotency, % of Blue-Color	Relative Biopotency, % of U S P XV
All-trans	1,818,000	1,818,000	100	1,818,000	100	100
2-mono- <i>cis</i>	1,818,000	1,301,000	72	1,370,000	75	105
6-mono- <i>cis</i>	1,818,000	1,263,000	69	413,000	23	33
2,6-di- <i>cis</i>	1,818,000	1,320,000	73	413,000	23	31

<sup>a</sup> From data on vitamin A acetates published by Robeson, *et al* (2), Ames, *et al* (3), and Embree, *et al* (4), calculated stoichiometrically for the palmitate ester, since none of the properties given is affected significantly by the ester form

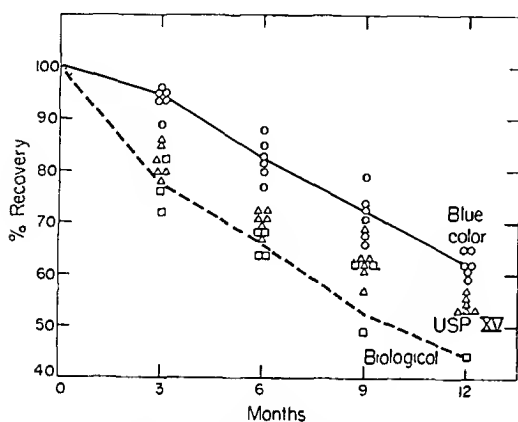


Fig 2—Vitamin A recovery in multivitamin drop preparations stored at 37°

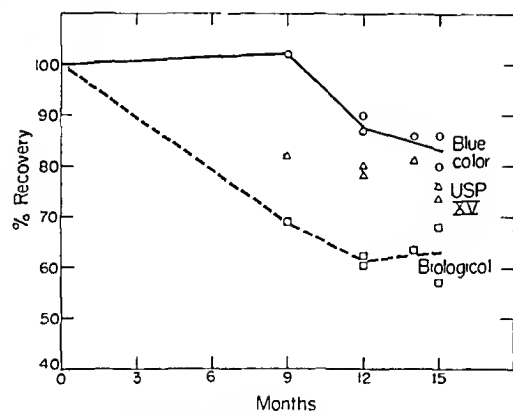


Fig 3—Vitamin A recovery in multivitamin drop preparations stored at 25°

in Table II. In interpreting these data, we consider that chemical destruction of vitamin A is most accurately measured by the change in the blue-color assay; and that the ratios of U S P XV potency to blue-color potency and of biopotency to blue-color potency are indicators of isomerization. Thus, the effect of isomerization, independent of chemical destruction, can be seen in Table III. Here the relative potencies are shown at each storage period. The U S P XV and biological "potencies" are expressed as a percentage of the blue-color "potency," and the biopotency is expressed as a percentage of U S P XV "potency." Results are shown both at 37 and 25° storage

TABLE II—% RECOVERY OF VITAMIN A IN AQUEOUS MULTIVITAMIN DISPERSIONS

	Months				
	3	6	9	12	15
37°					
Antimony Trichloride					
Blue-Color	94	82	72	62	
U S P XV	82	71	62	55	
Biological	77	66	53	44	
25°					
Antimony Trichloride					
Blue-Color			102	88	84
U S P XV			82	79	77
Biological			69	62	63

TABLE III—"RELATIVE POTENCY" OF VITAMIN A IN AQUEOUS DISPERSIONS

	Months				
	3	6	9	12	15
37°					
U S P XV/Antimony					
Trichloride Blue-Color	86	85	86	88	
Biological/U S P XV	94	92	84	80	
Biological/Antimony					
Trichloride Blue-Color	81	78	72	70	
25°					
U S P XV/Antimony					
Trichloride Blue-Color			83	89	92
Biological/U S P XV			78	76	77
Biological/Antimony					
Trichloride Blue-Color			65	68	71

Upon storage, the relative biopotency drops within the first few months of storage and then levels off at a value near 70% of the antimony trichloride blue-color value. Likewise, the relative biopotency drops and levels off at about 80% of the U S P XV value. It appears that most of the isomerization effect takes place during the first few months of storage.

Another indicator of isomerization is the reaction of vitamin A with maleic anhydride. This has been used (2, 4, 7) to determine the proportion of the total vitamin A present that has a *cis*-configuration at the 2-position. Expressed as "maleic value," its relationship to biopotency is the subject of a companion paper (8).

Table IV presents the averages of the maleic values obtained at 37° and at 25° for the different storage periods. Upon storage at 37°, the maleic value levels off at about 25%, while at room temperature it reaches 34%. It is still uncertain whether

TABLE IV.—MALEIC VALUE OF VITAMIN A IN AQUEOUS DISPERSIONS

	Months					
	0	3	6	9	12	15
37°	6	22	27	26	24	
25°	6			27	30	34

or not the final equilibrium is the same at 25° as a 37°

A third measure of isomerization depends on the infrared absorption curves of purified vitamin A aldehydes (9, 10, 11). Figure 4 shows the infrared absorption curves over a very narrow wavelength range for all-*trans*- and 6-mono-*cis*-vitamin A aldehydes. At 8.6  $\mu$  the all-*trans*-vitamin A has an absorption peak where the 6-mono-*cis*-isomer has an absorption minimum. The reverse occurs just below 8.75  $\mu$ . In the remainder of the infrared region, the absorption curves of these two isomers are nearly identical. The absorption curve for 2-mono-*cis*-vitamin A is similar to that of all-*trans* while the curve for 2,6-di-*cis*- is similar to that of the 6-mono-*cis*-isomer.

Two of the aqueous multivitamin drop preparations in the present study were examined by infrared absorption after storage for one year at room temperature. They were saponified, the unsaponifiable fractions oxidized to vitamin A aldehyde, and then purified for infrared analysis. Both were estimated (10) to contain 18% of their vitamin A in the 6-mono-*cis*- and 2,6-di-*cis*-forms. A third preparation was similarly examined after fifteen months' storage at room temperature. It was estimated to contain 21% combined 6-*cis*-isomers.

### CONCLUSIONS

All-*trans*-vitamin A isomerizes on storage in aqueous multivitamin drop preparations. The resulting equilibrium mixture contains isomers of much lower biological potency than all-*trans*- and 2-mono-*cis*-vitamin A. The relationships between the different "potencies" found by rat liver-storage bioassay, by U. S. P. XV spectrophotometric assay, and by antimony

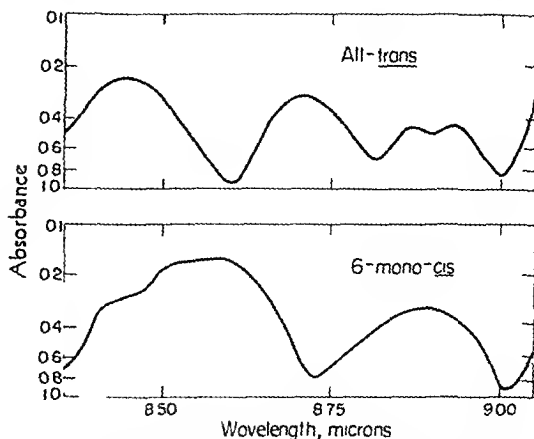


Fig. 4—Infrared absorption curves, partial, for all-*trans*- and 6-mono-*cis* vitamin A

trichloride blue-color assay indicate that the 6-mono-*cis*- and 2,6-di-*cis*-isomers are formed; their presence is confirmed by infrared absorption measurements. These effects are in addition to the chemical decomposition of vitamin A that takes place at the same time.

### REFERENCES

- (1) Pauling, L., *Fortschr. Chem. org. Naturstoffe*, **3**, 203(1939).
- (2) Robeson, C. D., Cawley, J., Weisler, L., Stern, M., Eddinger, C., and Chechak, A., *J. Am. Chem. Soc.*, **77**, 4111(1955).
- (3) Ames, S. R., Swanson, W. J., and Harris, P. L., *ibid.*, **77**, 4134(1955).
- (4) Embree, N. D., Ames, S. R., Lehman, R. W., and Harris, P. L., "Methods of Biochemical Analysis," Vol. IV, Interscience Publishers Inc., New York, N. Y., 1957, p. 43.
- (5) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1955.
- (6) Ames, S. R., and Harris, P. L., *Anal. Chem.*, **28**, 874(1956).
- (7) Robeson, C. D., and Baxter, J. G., *J. Am. Chem. Soc.*, **69**, 136(1947).
- (8) Ames, S. R., Swanson, W. J., and Lehman, R. W., *This Journal*, **49**, 366(1960).
- (9) Robeson, C. D., Blum, W., Dieterle, J., Cawley, J., and Baxter, J., *J. Am. Chem. Soc.*, **77**, 4120(1955).
- (10) Blum, W. P., and Stern, M. H., personal communication.
- (11) Brown, P. S., Blum, W. P., and Stern, M. H., *Nature*, **184**, 1377(1959).

# Estimation of the Biological Potency of Isomerized Vitamin A Palmitate in Aqueous Multivitamin Dispersions from Maleic Values\*

By STANLEY R. AMES, WILLIAM J. SWANSON, and ROBERT W. LEHMAN

The relative biopotencies on a blue-color basis and the maleic values of isomerized all-*trans*-vitamin A palmitate from stored aqueous multivitamin dispersions are related by the cubic regression equation: relative biopotency =  $99.5 - 0.2(MV) - 0.051(MV)^2 + 0.000768(MV)^3$ . Using this equation, the vitamin A biopotency of aqueous multivitamin dispersions can be computed from chemical data. This cubic equation relating the relative biopotency and maleic value was successfully applied to a number of mixtures of vitamin A isomers of both synthetic and natural origin. The presence of 6-*cis*-isomers of low biological potency in such mixtures of vitamin A isomers is indicated.

VITAMIN A PALMITATE, stored in aqueous multivitamin dispersions, has been reported by Lehman, *et al* (1), to isomerize to a mixture of isomers. Not only was 2-mono *cis*-(neo) vitamin A formed, but, in addition, substantial amounts of the 6 *cis*-isomers (6-mono-*cis*- and 2,6-di *cis*-) were formed. The degree of isomerization can be readily determined by bioassay of the aqueous dispersion. However, there is real need for a rapid chemical procedure to estimate the biopotency of a mixture of vitamin A isomers. The data in this communication further substantiate the report of Lehman, *et al* (1), that isomers of low biological potency (6-*cis* isomers) are formed in aqueous multivitamin dispersions. Relative biopotency and maleic value are related by a cubic equation.

## EXPERIMENTAL

**Preparations Tested.**—Some of the aqueous multivitamin dispersions were prepared in the laboratory as previously described (1). Others were samples of commercial products of known composition and length of storage. All samples had initially been prepared from all-*trans*-vitamin A palmitate.

**Maleic Value Determination.**—Maleic anhydride forms a condensation product of the Diels Alder type with those vitamin A isomers in which both the 2—3 and 4—5 double bonds are *trans*. The use of this reagent for the assay of neovitamin A in mixtures with all *trans*-vitamin A was described by Robeson and Baxter (2). The reaction of maleic anhydride with 6-mono *cis*-vitamin A was described by Robeson, *et al* (3). Details of the procedure

have been previously described by Embree, *et al* (4). Thus, the maleic value measures the amount of unreacted 2-mono *cis*- and 2,6 di-*cis*-vitamin A. The maleic value is computed from the following equation

$$\text{Maleic value (\%)} = \frac{R - R_1}{R_2 - R_1} \times 100$$

where  $R$  = recovery of vitamin A in the test sample. The constants  $R_1$  and  $R_2$  have been recently re-evaluated (5) for vitamin A esters  $R_1 = 20$  and  $R_2 = 93.4$ , for vitamin A alcohol  $R_1 = 11$  and  $R_2 = 89.5$ .

**Bioassay Procedure.**—The vitamin A biopotencies of aqueous multivitamin dispersions were determined by the slope ratio liver-storage procedure of Ames and Harris (6). In this procedure, rats are supplemented with 1,000 to 2,000 units of vitamin A during a one- to three day period. The livers are removed forty-eight hours after the final dose and analyzed for vitamin A by the procedure of Ames, Risley, and Harris (7). Some aqueous dispersions were administered by quantitative oral injection into the rat's stomach and compared with U S P vitamin A reference solution (8) similarly administered. In most cases, the aqueous dispersions were saponified and an oil solution of the nonsaponifiable matter administered orally by dropper and compared with nonsaponifiable fraction from the U S P vitamin A reference solution similarly administered.

In this report

Relative biopotency (%) =

$$\frac{\text{Biopotency}}{\text{Chemical potency by antimony trichloride (blue-color) determination}^1} \times 100$$

since the determination of vitamin A with antimony trichloride is the best measure of total vitamin A, irrespective of isomeric structure (4). The relative biopotencies for the four vitamin A isomers are all *trans*-, 100%, 2 mono-*cis*-, 75%, 6 mono-*cis*-, 23%, and 2,6 di *cis*-, 23%, as previously reported by Ames, *et al* (9).

<sup>1</sup> The U S P vitamin A reference solution was used as the standard for the antimony trichloride (blue color) determination (4) of vitamin A.

\* Received August 21, 1959 from the Research Laboratories of Distillation Products Industries, Division of Eastman Kodak Co., Rochester, N. Y.

Paper XVI of a series entitled "Biochemical Studies on Vitamin A."

The expert technical assistance of H. A. Risley, J. M. Dieterle, and W. T. Fisher of the Research Laboratories and H. W. Rawlings and G. H. Wait of the Manufacturing Control Laboratory greatly assisted the progress of this investigation.

Presented to the Scientific Section, A. P. A. Cincinnati meeting, August 1959.

RESULTS

A series of aqueous multivitamin dispersions of all-*trans*-vitamin A palmitate were stored for varying lengths of time. Maleic values and relative biopotencies on a blue-color basis were determined. The results of fifty-three analyses, as plotted in Fig. 1, indicated that the relative biopotency varied inversely with the maleic value. The linear regression of relative biopotency on maleic value was computed, but on inspection failed to fit the observed data for maleic values less than ten. However, the cubic regression of relative biopotency on maleic value showed the required curvature at low maleic values, and fit the data more closely than either the linear or quadratic regression. Coefficients for the quadratic and cubic terms are statistically significant, as shown in Table I. The equation for the cubic regression plotted in Fig. 1 is

Relative biopotency = 99.5 - 0.2(MV) - 0.051(MV)<sup>2</sup> + 0.000768 (MV)<sup>3</sup>

with standard deviation of the calculated relative biopotency minus observed relative biopotency equal to 3.55.

TABLE I.—SIGNIFICANCE OF THE CUBIC REGRESSION  
 $y = a + bx + cx^2 + dx^3$

Term	Coefficient	$\sigma$ (coefficient)	$t$ (coefficient)
Constant			
(a)	99.5	0.89	111.0 <sup>a</sup>
Linear (b)	0.2	0.33	0.491
Quadratic			
(c)	0.051	0.022	2.30 <sup>b</sup>
Cubic (d)	0.000768	0.000379	2.03 <sup>b</sup>

$df = 48. \quad p = 0.05. \quad t = 2.01.$

<sup>a</sup> Highly significant.  
<sup>b</sup> Significant.

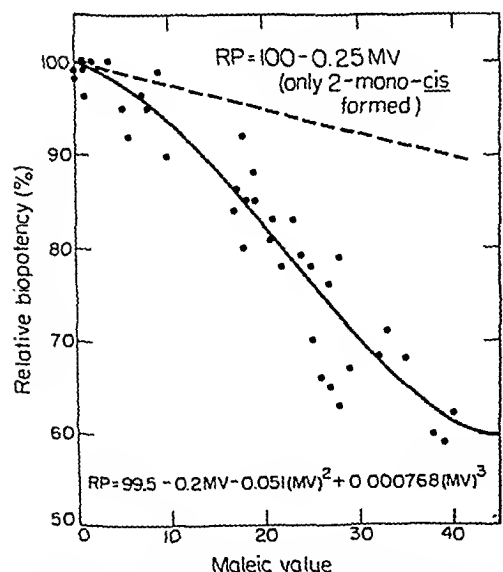


Fig. 1.—Relative biopotency (RP) vs. maleic value (MV) for all-*trans*-vitamin A isomerized in aqueous multivitamin dispersions.

The relative biopotency of a mixture of all-*trans*- and 2-mono-*cis*-vitamin A can readily be computed from maleic values by the linear equation plotted in Fig. 1. The bioassay results found with all-*trans*-vitamin A palmitate stored in aqueous multivitamin dispersions can not be explained by the isomerization of all-*trans*-vitamin A only to the 2-mono-*cis*-isomer. These data further substantiate the conclusions of Lehman, *et al.* (1), that in an aqueous dispersion all-*trans*-vitamin A isomerizes to a mixture of isomers including the 6-mono-*cis*- and 2,6-di-*cis*-isomers, which have low biological potency.

Several previous attempts to relate the relative biopotency with various chemical or spectral properties of the vitamin A isomers met with limited success. The use of least squares statistics to relate the relative biopotency and maleic value is empirical, but offers the best practical solution yet developed. The linear, quadratic, and cubic regressions of relative biopotency on maleic value were compared. The quadratic coefficient in the quadratic regression was not statistically significant, and this equation was therefore rejected. The cubic regression equation is preferred to the linear form because of both a better fit to the observed data at low maleic values and a lesser overall standard deviation of calculated minus observed relative biopotency. The cubic form of the regression equation suggests that initially, all-*trans*-vitamin A palmitate isomerizes more rapidly to the 2-mono-*cis*-isomer which has 75% of the biopotency of all-*trans*-vitamin A than to the 6-*cis*-isomers which have only 23% of the biopotency of all-*trans*-vitamin A.

This isomerization of all-*trans*-vitamin A palmitate in aqueous multivitamin dispersions appears to reach an equilibrium. Even after prolonged storage, the relative biopotency does not drop below 60-70% nor does the maleic value increase to values greater than 30-40%. Such an equilibrium mixture has been previously described by Ames, *et al.* (10), for vitamin A isolated from rat livers, and corresponds to a mixture of the four isomers in which the ratio of *trans*- to *cis*-isomer about each of the two double bonds, 2-3 and 6-7, is 2 to 1. Chemical isomerization of 2-mono-*cis*-vitamin A esters results in an isomer mixture with similar properties (3). Thus, a similar mixture of vitamin A isomers can be formed by isomerizing either all-*trans*- or 2-mono-*cis*-vitamin A.

APPLICATION TO OTHER ISOMER MIXTURES

The cubic regression equation described above has shown excellent results in predicting relative biopotencies from observed maleic values for vitamin A palmitate stored in aqueous multivitamin dispersions. Application of this equation to other mixtures of vitamin A isomers was tested. Preisomerized vitamin A palmitates were obtained by chemically isomerizing the all-*trans*-isomer by various procedures (2, 11). Twenty-seven such preparations were analyzed for both relative biopotency and maleic value. The results, plotted in Fig. 2, show excellent agreement with the cubic regression equation described above. Those preisomerized vitamin A palmitates with relative biopotencies and maleic values corresponding to an equilibrium

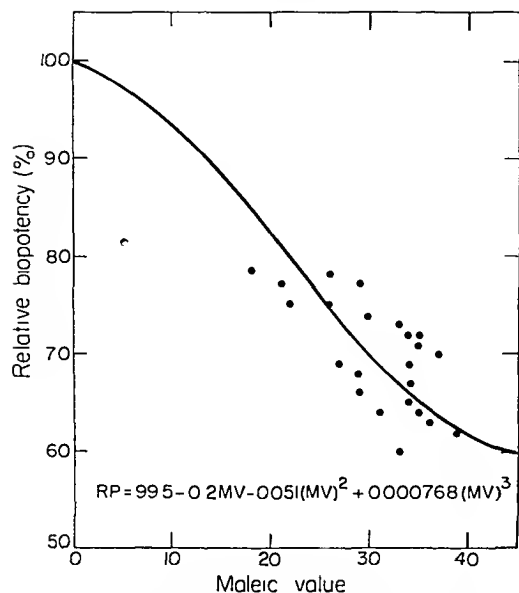


Fig. 2.—Relative biopotency (RP) vs maleic value (MV) for preisomerized vitamin A preparations

mixture of isomers exhibited no significant isomerization on storage in aqueous multivitamin dispersions (12)

Several naturally-occurring vitamin A oils have been found to contain substantial quantities of

6-*cis*-isomers (13). Such mixtures of vitamin A isomers have been isolated from fish-liver oils and lipid extracts of both rat and human livers. The relative biopotencies and maleic values for these preparations are correlated by the above cubic regression equation. These data suggest that the cubic regression of relative biopotency on maleic value may have general application to mixtures of vitamin A isomers from both synthetic and natural sources.

## REFERENCES

- (1) Leberman, R. W., Dieterle, J. M., Fisher, W. T. and Ames, S. R., *THIS JOURNAL*, 49, 363 (1960)
- (2) Robeson, C. D., and Baxter, J. G., *J. Am. Chem. Soc.*, 69, 136 (1947)
- (3) Robeson, C. D., Cawley, J. D., Weisler, L., Stern, M. H., Eddinger, C. C., and Cbechak, A. J., *ibid.*, 77, 4111 (1955)
- (4) Embree, N. D., Ames, S. R., Leberman, R. W., and Harris, P. L., "Methods of Biochemical Analysis," Vol. IV, Interscience Publishers Inc., New York, N. Y., 1957, p. 43
- (5) Ames, S. R., to be published
- (6) Ames, S. R., and Harris, P. L., *Anal. Chem.*, 28, 874 (1956)
- (7) Ames, S. R., Risley, H. A., and Harris, P. L., *ibid.*, 26, 1378 (1954)
- (8) U. S. P. Reference Standards, "Important Notice Regarding the U. S. P. Vitamin A Reference Solution," March 1, 1957
- (9) Ames, S. R., Swanson, W. J., and Harris, P. L., *J. Am. Chem. Soc.*, 77, 4134 (1955)
- (10) Ames, S. R., Swanson, W. J., and Harris, P. L., *Federation Proc.*, 16, 145 (1957)
- (11) Robeson, C. D., Blum, W. P., Dieterle, J. M., Cawley, J. D., and Baxter, J. G., *J. Am. Chem. Soc.*, 77, 4120 (1955)
- (12) Leberman, R. W., to be published
- (13) Brown, P. S., Blum, W. P., and Stern, M. H., *Nature*, 184, 1377 (1959)

# The Isolation of $\beta$ -Allocryptopine from *Argemone squarrosa* subsp. *squarrosa*\*

By T. O. SOINE and R. E. WILLETTE†

An examination of the alkaloidal content of *Argemone squarrosa* subsp. *squarrosa* has yielded approximately 1 per cent of  $\beta$ -allocryptopine as the major alkaloid, if not the only one. The alkaloid has been identified by conventional derivatives as well as by comparison of its infrared spectrum with that of its physical isomeride,  $\alpha$ -allocryptopine. Pharmacological testing has indicated that the alkaloid is relatively inactive except for its known ability to suppress auricular fibrillation.

STUDIES in these laboratories previously have involved the investigations of *Argemone hispida* (1, 2) and *A. munita* subsp. *rotundata* (Rydb.) (3) with specific reference to their alkaloidal content. These studies led to the isolation of three alkaloids provisionally named argemonine, norargemonine, and rotundine. Unpublished studies since that time have led to the belief that

these alkaloids are representatives of the isoquinoline group and are specifically of the aporphine type. It was of interest, therefore, to examine another species, namely *Argemone squarrosa* subsp. *squarrosa* to see whether it too contained alkaloids derived from the aporphine type of isoquinoline alkaloids.

A preliminary assay indicated a rather favorable alkaloidal content. Ethanolic extractions made in a Soxhlet and a Lloyd apparatus, however, afforded a lower alkaloidal yield than that

\* Received February 29, 1960, from the College of Pharmacy, University of Minnesota, Minneapolis 14.

† Rowell Laboratories Fellow, 1957-1958 and 1958-1959. Present address: Division of Pharmacy, Ferris Institute, Big Rapids, Mich.

possible according to the assay. A much more satisfactory yield was obtained by utilizing modified Prollius fluid in a percolator of our own design. In all cases, the total yield was comprised almost entirely of a single alkaloid which was conclusively identified as  $\beta$ -allocryptopine, by means of physical properties, derivatives, and infrared spectra.

With respect to infrared comparisons, it was noted that there were minor differences in oil mull preparations between the dimorphic forms. These differences, due to crystal structure, were eliminated by comparing the two samples in solution, in which case the dimorphism was no longer operative.

Allocryptopine has been isolated previously from various sources (4), and from the *Argemone* genus has been reported from *A. mexicana* (5). The alkaloid is dimorphic, the lower melting  $\alpha$ -form, being known also as  $\beta$ -homochelidonine, and the higher melting  $\beta$ -form, being known also as  $\alpha$ -fagarine and  $\gamma$ -homochelidonine.

## PHARMACOLOGY

The alkaloid has received considerable attention pharmacologically (6, 7). It has been claimed to be five times as active as quinidine in suppressing auricular fibrillation (6, 8) and has found some use in South America for this purpose (9). Unfortunately, it has a tendency to initiate ventricular fibrillation which imposes serious limitations on its use.

Prior to our identification, pharmacological tests<sup>1</sup> indicated that the base was relatively inactive. With respect to the central nervous system the drug failed to affect the qualitative behavior of the dog at 20 mg./Kg. or the spontaneous activity of the rat at a dose of 81 mg./Kg. At a dose of 300 mg./Kg. in the mouse,  $\frac{1}{3}$  died and excitement, tremor, and convulsions were evident. The base was also inactive with respect to the blood pressure, diuretic action, anti-inflammatory action (iritis), ankle edema, and the tracheal chain test (bronchial relaxation).

## EXPERIMENTAL

**Material.**—The dried, whole supraterranean plant of *Argemone squarrosa* subsp. *squarrosa*,<sup>2</sup> collected in southeastern Colorado in the Las Animas area, was ground to a coarse powder in preparation for extraction.

**Extraction.**—The ground plant (15.45 and 15.48 Gm.) was assayed according to the nux vomica assay procedure (10) and gave 1.39% and 1.64%, respectively, total alkaloidal content. The ground plant (400 Gm.) was then extracted for one hun-

dred and sixteen hours with 95% ethanol in a Soxhlet apparatus until negative to Mayer's T. S. The extract was concentrated to a syrup *in vacuo*, dissolved in methylene chloride, extracted with 10% hydrochloric acid, basified and extracted with methylene chloride, which upon concentration yielded 3.5 Gm. (0.87%) of total alkaloid. When the ground drug (6 Kg.) was extracted in a Lloyd apparatus and the extract processed in like manner, only 25 Gm. (0.42%) was obtained.

Inasmuch as the assay utilized an ether:chloroform:ammonia solvent, the ground plant (3 Kg.) was extracted by shaking it with 5 L. of modified Prollius fluid (ether:chloroform:ethanol:concentrated ammonium hydroxide, 250:55:25:10) in a 5-gallon bottle. It was allowed to stand overnight, the mouth provided with a suitable filter, the bottle inverted, air pressure applied to the bottom, and the contents allowed to percolate. This was repeated five times before a negative test was obtained with Mayer's T. S. This extract was concentrated to a syrup on a steam bath with the aid of a stream of air. It was dissolved in Skellysolve B and thoroughly mixed with 10% sulfuric acid, evaporating the organic solvent with a stream of air after it was allowed to rise to the top. This undisturbed mass was frozen and the acid-insoluble material separated from the top of the frozen acid solution which was very clear upon melting. The acid-insoluble residue was extracted twice more in this manner until negative to Mayer's T. S. This method proved far more satisfactory than extraction in a separatory funnel, in which case emulsions formed with considerable ease. The acid extract was alkalinized with concentrated ammonium hydroxide and, upon standing overnight, large masses of crystals had formed, which were filtered off, yielding 20 Gm. (0.67%) of  $\beta$ -allocryptopine. The filtrate was extracted with ten 250-ml. portions of methylene chloride, which upon concentration yielded a further 11 Gm. (0.37%) of alkaloid, from which 10 Gm. of  $\beta$ -allocryptopine was separated by crystallization, giving a total of over 1% of  $\beta$ -allocryptopine from the plant. The residues are being investigated for further alkaloids.

**Identification of  $\beta$ -Allocryptopine.**—The isolated alkaloid responded to all the usual color tests for the allocryptopines (11) such as a deep reddish-violet color with concentrated sulfuric acid and a positive methylenedioxy test. The base was recrystallized several times from 95% ethanol, dried at 100° *in vacuo* overnight, the long white needles melting at 169–171° [(lit. m. p. 170–171° (4c, 11)]. A mixed melting point with an authentic sample of  $\alpha$ -allocryptopine (m. p. 162°), graciously supplied by R. H. F. Manske, gave depression to 166°. When this melt was allowed to cool and recrystallize, it melted at 170°. All efforts to convert either sample back to the  $\alpha$ -form failed.

**Anal.**—Calcd. for  $C_{21}H_{23}NO_3$ : C, 68.28; H, 6.28; N, 3.79. Found: C, 68.30; H, 6.57; N, 3.75.

The infrared absorption spectra of the base (Fig. 1a), the sample of  $\alpha$ -allocryptopine supplied by Manske and  $\beta$ -homochelidonine<sup>3</sup> (Fig. 1b) were determined using a Perkin and Elmer spectrophotometer,

<sup>1</sup> All melting points were determined with a Kofler Micro-melting point apparatus and are corrected.

<sup>2</sup> The authors acknowledge and appreciate the kind cooperation of G. D. Searle and Co., Chicago, Ill., in the pharmacological testing.

<sup>3</sup> The authors are indebted to Dr. Gerald B. Ownbey, Botany Department, University of Minnesota, for the identification of the plant material. The nomenclature employed in this paper is in accord with that in Ownbey, G. B., *Mem. Torrey Bot. Club*, 21, (1950). A herbarium specimen has also been deposited in the archives of the Botany Department University of Minnesota.

<sup>4</sup> All analyses were carried out by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minn. Obtained from L. Light & Co. Ltd., Colnbrook, England.

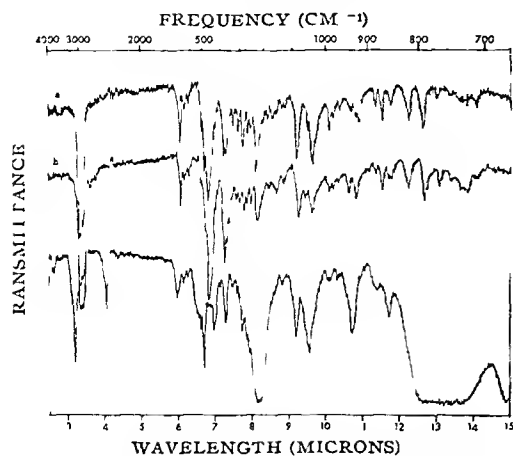


Fig 1—Infrared spectra of: (a),  $\beta$ -allocryptopine, Nujol mull; (b),  $\alpha$ -allocryptopine (Manske) or  $\beta$ -homochelidonine (Light), Nujol mulls; (c),  $\beta$ -allocryptopine or  $\beta$ -homochelidonine in chloroformic solution

**Model 137** They showed some minor differences in oil mull preparations, but gave identical spectra (Fig 1c) in chloroformic solution

**Hydrochloride.**—This salt was prepared by extracting a dilute hydrochloric acid solution of the base with chloroform, which upon concentration gave very fine needles of the hydrochloride. Recrystallized from ethanol, it began to turn yellow at  $170^\circ$  and decomposed at  $195^\circ$  [lit m p. 190–192° (decompn) (9) and m p  $175^\circ$  (11)] Upon drying at  $100^\circ$  *in vacuo* overnight, it melted at  $189$ – $190^\circ$ . It contains a molecule of solvent of crystallization (9)

**Anal**—Calcd for  $C_{21}H_{23}NO_5 \cdot HCl \cdot C_2H_5OH \cdot C$ , 61.10, H, 6.64. Found: C, 60.99; H, 6.59

**Methiodide.**—This derivative was prepared in the usual manner. Recrystallized from ethanol, the fine, white needles melted at  $190$ – $205^\circ$  (decompn) [lit m p 205–206° (9)] Upon drying at  $100^\circ$  *in vacuo* overnight, they melted at  $204$ – $205^\circ$

**Anal**—Calcd. for  $C_{21}H_{23}NO_5 \cdot CH_3I$ : C, 51.60, H, 5.08. Found: C, 51.88; H, 5.42

**Aurichloride.**—This salt was prepared by precipitating the base with 0.2 *N* auric chloride T S. It crystallized very slowly from ethanol giving characteristic warty, dark red crystals, melting at  $187$ – $187.5^\circ$  [lit B  $HuCl_4$ , m p  $187$ – $192^\circ$  (9)]

**Picrate.**—This derivative was prepared in the usual manner and recrystallized from anhydrous ethanol, m p  $208.5$ – $209.5^\circ$  [lit m p  $208$ – $209^\circ$  (4c)]

**Anal**—Calcd for  $C_{21}H_{23}NO_5 \cdot C_6H_3N_3O_7$ : C, 54.19, H, 4.44. Found: C, 54.18; H, 4.38

## SUMMARY

1  $\beta$ -Allocryptopine has been isolated from *Argemone squarrosa* subsp. *squarrosa* in approximately 1 per cent yield.

2 The alkaloid has been identified by means of derivatives and comparison of its infrared spectrum with those of known samples of its physical isomeride,  $\alpha$ -allocryptopine.

3. Some pharmacological data, in addition to that already in the literature, have been reported

## REFERENCES

- (1) Soine, T. O., and Gisvold, O., *THIS JOURNAL*, 33, 185 (1944)
- (2) Schermerhorn, J. W., and Soine, T. O., *ibid*, 40, 19 (1951)
- (3) Kier, L. B., and Soine, T. O., *ibid*, 49, 187 (1960)
- (4) a, Manske, R. H. F., and Holmes, H. K., "The Alkaloids," Vol. IV, Academic Press, New York, N. Y., p. 159; b, Deulofeu, V., Labriola, R., and DeLanghe, J., *J. Am. Chem. Soc.*, 64, 2326 (1942); c, Deulofeu, V., Labriola, R., and Benzaghi, B., *J. Org. Chem.*, 12, 217 (1947)
- (5) Slavikova, L., and Slavik, J., *Chem. listy* 49, 1546 (1955); *Chem. Abstr.* 50, 4990 (1955)
- (6) DiPalma, J. R., Lambert, J. J., Reiss, R. A., and Schultz, J. E., *J. Pharmacol. Exptl. Therap.*, 98, 251 (1950)
- (7) Alles, G. A., and Ellis, C. H., *ibid*, 104, 253 (1952)
- (8) Barlow, R. B., "Introduction to Chemical Pharmacology," John Wiley & Sons, New York, N. Y., 1955, p. 299
- (9) Redemann, C. E., Wisegarver, B. B., and Alles, G. A., *J. Am. Chem. Soc.*, 71, 1030 (1949)
- (10) "The National Formulary," 10th ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 400
- (11) Henry, T. H., "The Plant Alkaloids," 4th ed., The Blakiston Co., Philadelphia, Pa., 1949, p. 301.

# The Application of Alternating Current Polarography in the Determination of Phenobarbital in Dosage Forms\*

By N. G. LORDI, E. M. COHEN, and B. L. TAYLOR

The technique of alternating current polarography has been applied to phenobarbital, using a relatively simple modification of the Sargent model XXI polarograph. Distinctive analytically useful waves were obtained in pH 8 borate buffer containing 1 M potassium nitrate. Phenobarbital was determined in both liquid and solid dosage forms with an accuracy of 2 per cent by dilution of the sample and subsequent comparison of the wave scanned in buffer to that of a standard. Results indicated that the barbiturate could be determined directly in the presence of a wide variety of drugs without the necessity for isolation from the dosage form.

PRACTICAL METHODS for the determination of phenobarbital in its dosage forms have been reported by many workers. These may be conveniently divided into three groups, the first being gravimetric methods which involve isolation of the barbiturate from the dosage form and its subsequent weighing, an example of which is the present official U. S. P. method. The second group includes nonaqueous titrations (1, 2) using a variety of reagents and solvents which generally require prior extraction of the barbiturate from its dosage form. More recently, Vincent and Blake (3) effected the necessary separation of barbiturates from dosage forms with ion exchange resins prior to titration. The third group includes those methods which involve the direct titration of phenobarbital with a metal reagent which reacts with the barbiturate forming a weakly ionized or insoluble salt. Bodin (4) titrated phenobarbital potentiometrically in elixirs, parenteral solutions, capsules, and tablets with silver nitrate. Kalvoda and Zyka (5) employed mercuric nitrate in an amperometric technique using the dropping mercury electrode to determine phenobarbital in tablets and other solid mixtures. Cohen (6) has recently successfully titrated phenobarbital in elixirs with mercuric acetate, using either potentiometric or amperometric methods of end point detection.

In recent years the technique of alternating current polarography has come under close scrutiny as a means of extending the analytical usefulness of the polarographic method (7, 8). In this technique, a small sinusoidal alternating voltage is superposed on the direct potential normally applied to the dropping mercury electrode in conventional polarography. The alter-

nating component of the current produced is measured and recorded as a function of the applied direct potential. The resulting graphs are referred to as a. c. polarograms. The most significant feature of these plots is observed when substances which are reversibly reduced are studied. A maximum alternating current (summit current) is exhibited at the direct potential (summit potential) which corresponds to within  $\pm 50$  millivolts of the half-wave potential of the conventional polarogram. A. c. polarograms thus appear, at least in form, to be the first derivative of the d. c. polarogram.

Among the advantages a. c. polarography has over d. c. polarography is that oxygen need not be expelled from the solution to be analyzed, since substances which are irreversibly reduced and not adsorbed on the surface of the electrode do not exhibit a. c. polarograms. Thus, this technique may be used as a test for reversibility in redox processes. Other advantages include increased sensitivity and ease of separation of waves which occur at potentials close to one another.

In addition to substances which undergo reversible reduction or oxidation, solutions of surface-active materials produce wave-shaped current-voltage curves under a. c. conditions which have their origin in adsorption processes occurring at the electrode surface. Breyer and Hacobian (9) have termed this type of electrode process "tensammetry." In their words (10), tensammetric waves are a consequence of "... adsorption-desorption equilibrium at the electrode-solution interface without electron transfer." Breyer and Hacobian (11) have also recently applied tensammetry to the estimation of tungstate ion.

Experiments with phenobarbital, using the technique of alternating current polarography, revealed that it exhibited distinctive waves.

\* Received August 21, 1959, from the College of Pharmacy, Rutgers—The State University, Newark, N. J. Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.



The purpose of this report is to describe the properties of these waves and to indicate an approach to their analytical utilization in pharmaceuticals.

## EXPERIMENTAL

**Apparatus.**—The method of converting the Sargent model XXI polarograph to record a. c. polarograms, as originally described by Miller (12), was modified. The recording section of the Sargent polarograph is essentially a potentiometer in which a d. c. signal is converted into an a. c. signal which is in turn amplified and balanced against a standard reference signal. For this work the converter was removed from the amplifier and replaced by a six-prong plug with pins 3 and 4 shorted. This changed the recording section of the instrument from a d. c. measuring device into a high gain a. c. measuring device. The additional circuitry required is diagrammed in Fig. 1.

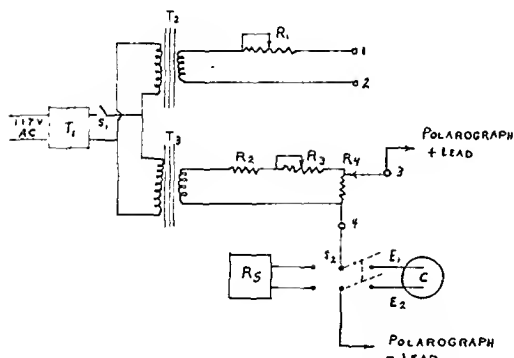


Fig. 1.—Electrical circuitry required for operation of Sargent model XXI polarograph as an a. c. polarograph.

C, polarographic cell;  $E_1$ , saturated calomel electrode;  $E_2$ , dropping mercury electrode;  $R_1$ , 5000 ohm potentiometer;  $R_2$ , 200 ohm,  $\frac{1}{2}$  watt resistor;  $R_3$ , 100 ohm potentiometer;  $R_4$ , 10 ohm potentiometer;  $R_5$ , decade resistance box;  $S_1$ , SPST switch;  $S_2$ , DPDT switch;  $T_1$ , constant voltage transformer, 115 volts a. c.;  $T_2$ , filament transformer; primary, 115 volts a. c.;  $T_3$ , secondary, 2.5 volts a. c.

The output of one of the filament transformers (terminals 1 and 2 on the diagram) was connected across the terminals normally occupied by the 11½ volt working cell in the recorder. These terminals must be connected so that the recorder will read zero with the cell leads open and be driven upscale when the cell leads are shorted. This functioned to introduce an alternating voltage 180 degrees out of phase with the signal to be measured. Provision was made for substitution of a decade resistor in place of the polarographic cell for purposes of calibration. In addition, a 500-microfarad, 25-volt, electrolytic capacitor was fixed in position in the polarographic section between the movable arm of the slide wire and the fixed arm of the sensitivity selector. This capacitor was necessary to shunt the a. c. signal around the slide wire to prevent changing the resistance to a. c. as the resistance of the polarograph slide wire was changed.

The presence of this capacitor did not affect the operation of the instrument as a d. c. polarograph.

All controls functioned as when the polarograph was operated as a d. c. device except the damping switch, which was, of necessity, kept in the off position. No attempt was made to calibrate the scale of the recorder in terms of absolute current. Rather the current scale used was expressed in arbitrary units obtained by multiplying the number of recorder chart scale divisions by the sensitivity setting of the instrument. In this work, the voltage across  $R_4$  was adjusted by means of  $R_3$  to a value of 100 millivolts r. m. s. The calibration point was selected to give optimum response at a sensitivity setting of 0.1 on the instrument. When  $R_4$  was adjusted to give an output of 30 millivolts r. m. s.,  $R_1$  was set so that, when a resistance of 1,000 ohms was substituted for the cell at a sensitivity setting of 0.1, the pen was deflected to exactly 15.0 on recorder scale. After each run this setting was adjusted to allow for the small amount of d which was observed to occur.

A conventional H-type cell (13) was employed with a 1.5 M potassium nitrate agar plug to minimize diffusion of chloride into the cell from reference electrode (a saturated calomel electrode). The characteristics of the dropping mercury electrode capillary (length: 9 cm.) were such that when operated under a pressure of 39 cm. of mercury, the drop time at 25° was 2.57 seconds in 1 KNO<sub>3</sub> at 0 applied potential and  $m$  was 3.26 n sec.<sup>-1</sup>

No exact theoretical interpretation could be made of the a. c. polarograms obtained with the apparatus described (8), since no provision was made for maintaining a constant a. c. voltage across the mercury-solution interface and no attempt was made to correct for the series resistance of the cell. However, since the intent behind this work was to develop an empirical analytical method for phenobarbital, the additional instrumentation required was not deemed essential to the achievement of this purpose.

The Beckman model G pH meter was employed for all measurements.

**Reagents.**—Phenobarbital U. S. P. (m. p. 177.5° uncorrected), was recrystallized twice from alcohol-water. Phenobarbital sodium U. S. P., was dried for several hours at 110° prior to use. The buffer used in this work consisted of 2.5 M potassium nitrate and 0.125 M boric acid to which 0.012 M sodium hydroxide was added. A 2.5-fold dilution of this buffer had a pH of 7.9. Reagent grade chemicals were employed unless otherwise stated.

**Preliminary Experiments.**—Initial efforts were directed towards determining those conditions under which the optimum phenobarbital wave could be elicited. Parameters evaluated were scanning rate, pH, ionic strength, drop time, magnitude of applied a. c. voltage, and temperature. Most experiments were carried out at a room temperature of 25.0–25.5°, using 30 millivolts r. m. s. applied a. c. Scanning rates of 1.23 and 0.62 millivolts per second were used. When quantitative results were desired a lower scanning rate was used, since true values

<sup>1</sup> Beckman chart No. 6353-N.

<sup>2</sup> Beckman model 1170-71.

summit current could only be obtained at low scanning rates or when the instrument was operated manually (14). Furthermore, the maxima of the current oscillations were used as the basis for reference in measuring current.

The waves of both phenobarbital and sodium phenobarbital were first evaluated in 1 *M* potassium nitrate. Typical waves obtained are shown in Fig. 2. It was noted that at low concentrations the waves of both the acid and salt forms of the barbiturate were nearly identical, while an eight-fold increase in concentration caused significant changes both in wave shape and current magnitude. This was to be expected, since  $10^{-4}$  *M* sodium phenobarbital is, for all practical purposes, completely hydrolyzed in the potassium nitrate media.

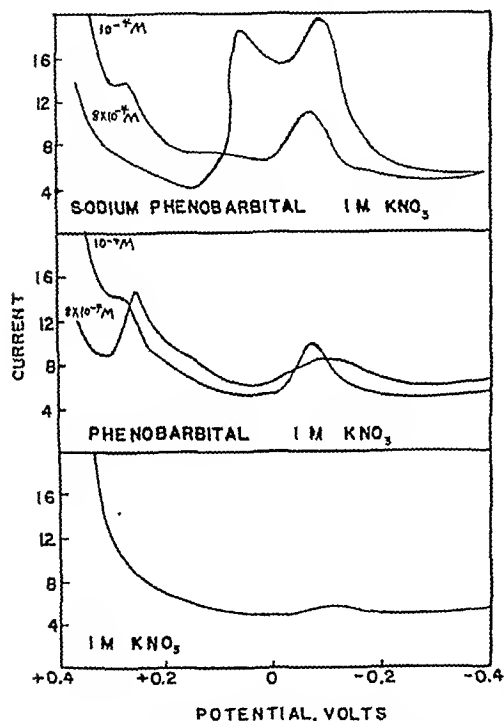


Fig. 2.—A. c. polarograms of phenobarbital and its sodium salt in 1 *M*  $\text{KNO}_3$

The pH of a solution, 1 *M* in potassium nitrate and initially containing 0.05 *M* boric acid and  $10^{-4}$  *M* phenobarbital to which 0.05 *M* sodium hydroxide had been added, was varied by the addition of increments of 2 *M* perchloric acid. The waves were scanned after the addition of each increment of acid. The necessity for pH control can be seen upon examination of Fig. 3. Below pH 7 the phenobarbital wave had disappeared. Optimum sensitivity, i. e., maximal summit current, was attained in the vicinity of pH 8. An increase in pH above this point tended to reduce both the sensitivity and resolution (degree of separation from the residual current) of the wave.

Reduction in the total ionic content of the supporting electrolyte also diminished both the sensitivity and resolution of the wave, as well as in-

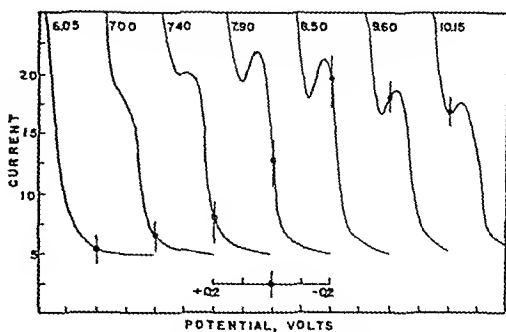


Fig. 3.—pH dependence of phenobarbital wave in borate buffer: 1 *M*  $\text{KNO}_3$  and  $10^{-4}$  *M* phenobarbital.

creased the magnitude of the residual or capacitive current which was a result of the charging of the mercury drop by the supporting electrolyte. However, sensitivity was again reduced at ionic strengths greater than 1.

The current was a function of the applied a. c. voltage, an increase in voltage tending to increase the current. However, increases in voltage to values greater than 30 mv. r. m. s. diminished the resolution of the waves. Reduction in voltage to a minimum of 10 mv. r. m. s. tended to improve resolution, especially at low concentrations. In the vicinity of room temperature the summit current was observed to fluctuate less than 0.5% per degree, tending to increase with temperature. The dropping rate was also observed to affect summit current, a change in drop time from 2.0 to 4.8 seconds increasing the current about 20%.

Further experiments indicated that the decomposition products of phenobarbital, obtained by heating a solution of the sodium salt in the presence of a tenfold excess of sodium hydroxide, produced no waves. This established the validity of using a. c. polarography as a means of assaying for phenobarbital.

**Effect of Phenobarbital Concentration.**—Typical phenobarbital waves recorded in pH 8 borate buffer are illustrated in Fig. 4. In order to establish the concentration dependence of the wave form, the summit current corrected for the residual current of the blank, i. e., the peak current, was plotted as a function of concentration. The resultant curve (Fig. 5) was nonlinear, an analytically useful standard curve being obtained only at concentrations lower than  $10^{-4}$  *M*. No significant changes in summit current were observed when the concentration of barbiturate was increased to 0.01 *M*.

**Procedure for Assay of Liquid Dosage Forms. Elixirs and Parenteral Solutions.**—A suitable aliquot of solution, containing about 20 mg. of phenobarbital, was diluted to volume in a 100-ml. volumetric flask with distilled water. One- or two-milliliter aliquots of this dilution were added to 10 ml. of buffer concentrate in a 25-ml. volumetric flask and brought to volume with distilled water. Each sample was scanned at a rate of 0.62 mv. sec.<sup>-1</sup> and the peak currents computed from the resulting waves. The concentration of barbiturate in the dosage form was calculated from an appropriate standard curve.

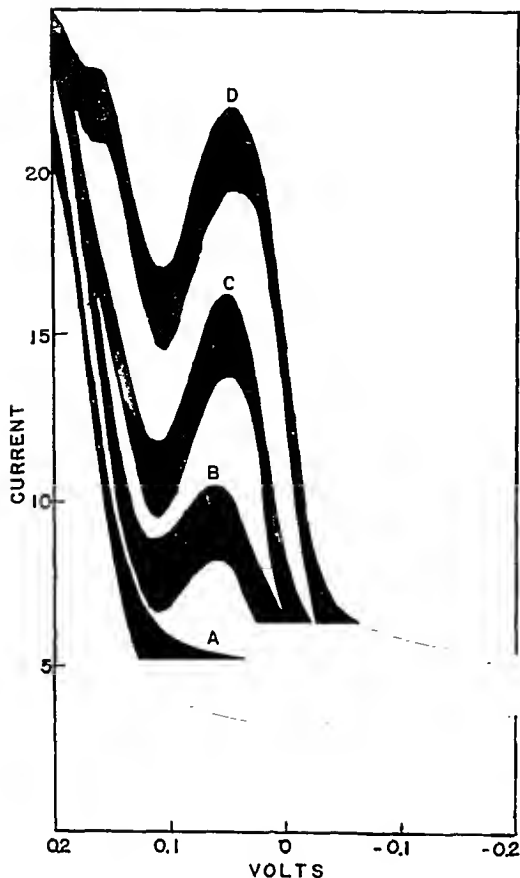


Fig. 4.—A. c. polarograms of phenobarbital in borate buffer: pH 7.9 and 1 *M* KNO<sub>3</sub>. Phenobarbital concentration: A, none; B,  $2 \times 10^{-6}$  *M*; C,  $5 \times 10^{-6}$  *M*; and D,  $10^{-4}$  *M*.

**Procedure for Assay of Solid Dosage Forms: Powders, Capsules, and Tablets.**—Accurately weighed, uniformly mixed samples containing approximately 20 mg. of phenobarbital were added to 10 ml. of alcohol U. S. P. in a 100-ml. volumetric flask. The mixture was agitated to extract the barbiturate from the solid before diluting to volume with distilled water. The addition of alcohol was omitted if the barbiturate was present as a water-soluble salt. Where necessary, the resultant mixture was centrifuged to remove insoluble matter before 1- or 2-ml. aliquots were taken for analysis in the manner already described.

The results obtained for the recovery of phenobarbital from some typical dosage forms are summarized in Table I.

**Effect of Foreign Substances on the Phenobarbital Wave.**—A study was made of the possible interference of other drugs which are commonly formulated with phenobarbital and certain other substances on the wave form. The phenobarbital wave was scanned in the buffer at a concentration of  $10^{-4}$  *M* in the presence of a ten-fold excess of the drug. The results indicated that the method was not applicable to the determination of the barbiturate in the presence of caffeine, diphenylhydantoin, aminophylline, theobromine sodium salicylate, thi-

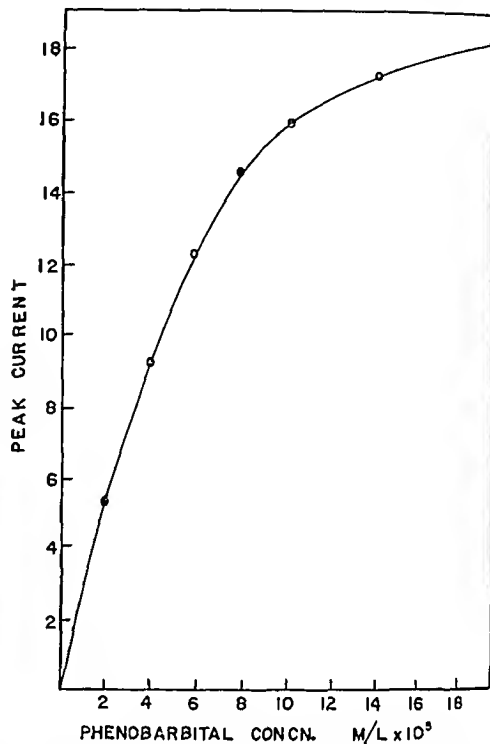


Fig. 5.—Concentration-peak current standard curve of phenobarbital in borate buffer: pH 7.9 and 1 *M* KNO<sub>3</sub>.

TABLE I.—ANALYSIS OF PHENOBARBITAL IN DOSAGE FORMS

Dosage Form	No. Samples	Recovery, %
Phenobarbital elixir, U. S. P. XV	8	101.0 $\pm$ 0.6
Phenobarbital sodium injection <sup>a</sup>	6	99.5 $\pm$ 1.4
Tablet mixture <sup>b</sup>	9	98.7 $\pm$ 0.8
Lactose dilution <sup>c</sup>	6	99.3 $\pm$ 1.7

<sup>a</sup> Propylene glycol solution containing 20 mg. of phenobarbital sodium per ml.

<sup>b</sup> Tablet formulation described by Bodin (4) containing 15 mg. of phenobarbital in each 200 mg.

<sup>c</sup> 1:10 trituration of phenobarbital in lactose.

amine hydrochloride, bromide, and iodide. These substances tended to distort the wave and/or change the character of the residual current. Aspirin, phenacetin, sodium salicylate, atropine sulfate, ephedrine sulfate, and chloride did not interfere. All the halides produced distinctive waves in the absence of phenobarbital, the summit potential of only chloride being significantly more positive (0.1 volts) than that of phenobarbital.

Concentrations of alcohol and propylene glycol less than 1% did not alter the peak current of phenobarbital significantly, while higher concentrations tended to reduce sensitivity as well as increase residual current. Polyethylene glycol 400 interfered in concentrations as low as 0.5%, tending to increase the residual current greatly. Strong surface active materials, e. g., sodium stearate and methylcellulose, depressed the peak current to a significant extent.

## DISCUSSION

The experimental data indicated that the successful application of alternating current polarography to the determination of phenobarbital in dosage forms depended, in part, upon the high dilution factors employed which minimized the effect of diluents, excipients, flavors, colors, and other foreign ingredients on the analytical phenobarbital wave. A case in point was phenobarbital elixir. The usual aliquot chosen for analysis was 5 ml, the sample employed to record the wave being a 250- to 500-fold dilution of the elixir. At these dilutions the components of the elixir base had no effect on the character of the wave. The particular tablet mixture studied contained, in addition to the barbiturate, magnesium stearate, acacia, starch, sucrose, and lactose, none of which affected the wave in the amounts normally present in tablets.

The degree of accuracy attainable, about 2%, was limited by the nature of the standard curve. The error involved in interpolation from the curve was further compounded by the necessity of using high dilution factors.

The principal advantages of the proposed method were its rapidity and the elimination of the need for isolation of the barbiturate from its dosage form. In many cases the method could be extended to mixtures containing interfering substances if blank corrections were made for the residual current and the calibration curve prepared in the presence of the interfering substances.

Unlike the d. c. polarographic diffusion current, the alternating current measured in a c. polarography is not dependent on concentration gradients near the electrode, but is a function of the concentration of active species at the electrode surface. As a consequence, when the electrode surface has become saturated, maximum peak current will have been attained. The form of the peak current-concentration curve will approximate that of an adsorption isotherm (Fig. 5).

The a. c. polarographic behavior of phenobarbital differed in several important respects from the behavior attributed by Breyer and Hacobian (9) to tensammetric processes. These investigators observed that the effect of an increase in temperature was to decrease peak current in the case of substances which exhibited positive temperature coefficients of solubility while the reverse was true in the case of those substances which exhibited negative solubility temperature coefficients (e. g. methylcellulose). They also observed that weak acids and bases (e. g. phenol and pyridine) were effective in producing tensammetric waves only in the undissociated state. In contrast to these observations it was found that phenobarbital showed greater peak current at elevated temperatures and was more effective in the salt form. Furthermore phenobarbital

showed no wave on the negative side of the electrocapillary zero, a characteristic of tensammetric processes, and produced well-defined waves at very low concentrations (e. g.,  $10^{-5}$  M).

Phenobarbital appeared to correspond more closely in behavior to the halides which exhibit a. c. polarographic waves referred to by Breyer and Hacobian (10) as "transition" waves. Unlike the true tensammetric wave, transition waves are a result of two processes: a polarographic step, involving interaction of the active species with the electrode to produce a surface-active product, and a tensammetric process, involving an adsorption-desorption equilibrium at the electrode surface. Thus, the a. c. waves of the halides are the result of reaction with mercury to form mercurous halide (i. e. the electrode reaction responsible for the existence of anodic d. c. polarographic waves) which is adsorbed on the mercury drop.

Phenobarbital is not polarographically reduced but does show an anodic wave (15). The a. c. polarographic behavior of phenobarbital, as indicated by these observations, appears to be a consequence of a reaction of phenobarbital with mercurous ion formed by a polarographic electrode reaction, the resultant mercurous phenobarbital being adsorbed at the mercury-solution interface.

Preliminary experiments were run with other barbiturates, i. e., pentobarbital, amobarbital, and secobarbital. All exhibited a. c. polarograms similar in form to those of phenobarbital. It is to be expected that the analytical technique described should be generally applicable to all barbiturates. A more extensive study of the a. c. polarography of the barbiturates will be the subject of a later paper.

## REFERENCES

- (1) Ryan, J. C., Yankowski, L. K., and Pifer, C. W. *This Journal*, **43**, 656 (1954).
- (2) Swartz, C. J., and Foss, N. E., *ibid.*, **44**, 217 (1955).
- (3) Vincent, M. C., and Blake, M. I., *ibid.*, **48**, 359 (1959).
- (4) Bodin, J. I., *ibid.*, **45**, 185 (1956).
- (5) Kalvoda, R., and Zyka, J., *Časopis českého lékařstva*, **63**, 1 (1950).
- (6) Cohen, E. M., "Three Instrumental Approaches to the Estimation of Phenobarbital in Elixir Dosage Form," M. Sc. Thesis, 1960, Rutgers University.
- (7) Breyer, B., Bauer, H. H., and Hacobian, S., *Australian J. Chem.*, **7**, 305 (1954).
- (8) Bauer, H. H., and Elving, P. J., *Anal. Chem.*, **30**, 334 (1958).
- (9) Breyer, B., and Hacobian, S., *Australian J. Sci. Research, Ser. A*, **5**, 500 (1952).
- (10) Breyer, B., and Hacobian, S., *Australian J. Chem.*, **6**, 186 (1953).
- (11) Breyer, B., and Hacobian, S., *Anal. Chim. Acta*, **16**, 497 (1957).
- (12) Miller, D. M., *Can. J. Chem.*, **34**, 942 (1956).
- (13) Kolthoff, I. M., and Lingane, J. J., "Polarography," Vol. 1, 2nd ed., Interscience Publishers, New York, N. Y., 1952, p. 362.
- (14) Thanka, N., Tamamushi, R., and Kodama, M., *Anal. Chim. Acta*, **20**, 573 (1959).
- (15) Brezina, M., and Zuman, P., "Polarography in Medicine, Biochemistry, and Pharmacy," Interscience Publishers, Inc., New York, N. Y. 1958 pp. 506-508.

# Study of the Stability of Secobarbital Sodium Solutions I\*

## Determination of Solubility of Secobarbital as a Function of Solvent and Hydrogen Ion Concentration

By J. H. UDANI and J. AUTIAN

The solubility of secobarbital in water at various temperatures was determined and from this information a plot was prepared,  $\log S$  vs.  $1/T$ . The linear relationship indicated the adherence of the curve to van't Hoff's equation. From the slope, the heat of solution was calculated and found to be 4358.0 cal./deg./mole. Solubility studies were also undertaken to determine the quantity of secobarbital which would dissolve in various binary solvent systems in which water was always the second solvent. The order of solubility increased from glycerin-propylene glycol-PEG 400-alcohol. Glycerin had very little solubilizing activity for secobarbital even in volume concentrations up to 50 per cent. In a three-component solvent system composed of alcohol: glycerin: water, the glycerin produced a synergistic effect on the dissolution reaction. Finally, the influence of pH on solubility of secobarbital in water alone and in several mixed solvents was ascertained, and the results plotted as solubility curves. It was also demonstrated that alcohol, propylene glycol, glycerin, and PEG 400 suppressed ionization of secobarbital in aqueous solutions. All of the solvents except PEG 400 showed a linear relationship in the solvent concentration studied.

STUDIES on aqueous solutions of the barbiturates indicate that pH and temperature will have an appreciable effect upon the stability of the drug. These studies further elucidate that as the hydrogen ion concentration and temperature are increased, the degradation proceeds rapidly. In order to circumvent the use of the salt of the particular barbituric acid, the acid form of the drug is employed with the addition of alcohol, propylene glycol, polyethylene glycols, or other agents to achieve dissolution of the relatively insoluble barbituric acid in water. The acidic pH thus insures greater product stability by decreasing the hydrolysis reaction known to occur in the alkaline pH range (1-3).

In this study, secobarbital sodium was selected as the barbiturate to investigate. Before undertaking a vigorous investigation of secobarbital stability, however, it became necessary first to accumulate data on the solubility of the drug as a function of solvent and pH in order to design the stability experiments properly. The information reported in this paper describes the solubility phase of the study.

### EXPERIMENTAL

**Apparatus and Supplies.**—Leeds & Northrup pH meter (Cat. 7664); shaking apparatus in constant temperature bath; secobarbital and secobarbital sodium U. S. P.; absolute alcohol U. S. P.; pro-

pylene glycol U. S. P.; glycerin U. S. P.; polyethylene glycol U. S. P.; absolute methyl alcohol, R. G.; chloroform, R. G.; potassium hydroxide, R. G.; thymol blue solution (0.5% w/v in methyl alcohol).

**Assay of Secobarbital.**—To determine the secobarbital in the various samples quantitatively, a nonaqueous titration method was used. The assay employed chloroform as the solvent and a standard (0.1 N) solution of potassium hydroxide in absolute methyl alcohol as the titrant, using thymol blue solution as the indicator. The exact procedure for the assay is given by Chatten (4). This method was compared with an ultraviolet spectrophotometric assay suggested by Mattson (5) and found to agree within experimental errors.

**Solubility as Function of Temperature.**—An excess quantity of secobarbital was added to 50-ml. Erlenmeyer flasks containing 30 ml. of freshly distilled water, previously heated to the desired temperature. Three such solutions were prepared for each temperature studied. The flasks were then stoppered, placed into a constant temperature bath at the desired temperature, and agitated for a period of twenty-four hours. The solution from each flask was then quickly filtered and an aliquot (approximately 5 ml.) transferred to a weighing bottle of known weight. After the bottle containing the solution was weighed, the liquid was poured into a 125-ml. separatory funnel. The bottle was rinsed with three separate portions (5 ml.) of distilled water and the combined rinse water added to the separatory funnel. Fractions of chloroform (25 ml.) were then used to extract the secobarbital for assay. In all instances, the volume of chloroform was concentrated to 50 ml. before proceeding with the assay. In this experiment, solubility was determined at 20, 25, 30, 35, 40, 45, and  $50^\circ \pm 0.2^\circ$ . The results were based on the average of three samples for each temperature.

The data were plotted as  $\log S$  versus  $1/T$  and found to be linear in the range studied (Fig. 1). The

\* Received August 21, 1959, from the College of Pharmacy, University of Michigan, Ann Arbor.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

The authors wish to express their sincere appreciation to Dr. Ewald Rohrmann, Eli Lilly and Co., for the generous supply of secobarbital and secobarbital sodium.

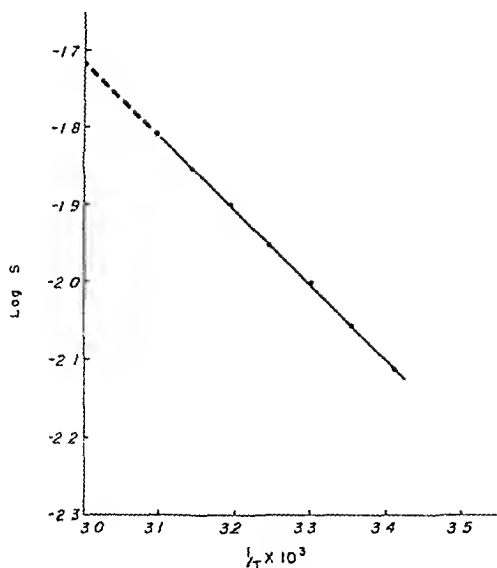


Fig. 1.—Solubility of secobarbital in water as function of absolute temperature.

linear relationship indicates its conformity to van't Hoff's equation

$$\log S = \frac{-\Delta H}{2.303 RT} + \text{constant} \quad (\text{Eq. 1})$$

where  $S$  is the solubility in moles per 1,000 Gm. of solvent at the absolute temperature  $T$ ,  $R$  is the gas constant, and  $\Delta H$  the heat of solution in calories per degree per mole. From the slope  $-\Delta H/2.303R$  the heat of solution ( $\Delta H$ ) was calculated and found to be 4358.0 cal./deg./mole.

**Solubility as Function of Solvent.**—Excess quantities of secobarbital were added to various concentrations of alcohol, propylene glycol, glycerin, and polyethylene glycol 400, water making up the remainder of the solvent. A 30-ml. sample for each concentration and solvent system was prepared, placed into a constant temperature bath at  $30^\circ \pm 0.2^\circ$ , and agitated for twenty-four hours. The secobarbital was extracted as described before for each sample and assayed for drug content. The results were based upon the average of three samples for each concentration and solvent system. Figure 2 presents the data in graphical form.

The solubility of secobarbital in a three-component solvent system was determined and the data are plotted in Fig. 3 as per cent solubility *versus* glycerin added to alcohol base. In each instance, the remaining quantity of solvent was water.

**Solubility as Function of pH.**—The desired quantity of secobarbital sodium (from a theoretical solubility curve) was weighed on a prescription balance and placed into a 50-ml. beaker containing 25 ml. of distilled water. Hydrochloric acid (1.0  $N$ ) was then added, dropwise, to adjust the pH to the requisite value, and the volume of the solution was brought up to 30 ml. with distilled water. This solution was then transferred to a 50-ml. Erlenmeyer flask, stoppered, and placed into a constant temperature bath at  $30^\circ \pm 0.2^\circ$ . A number of such solutions were prepared, each at a differ-

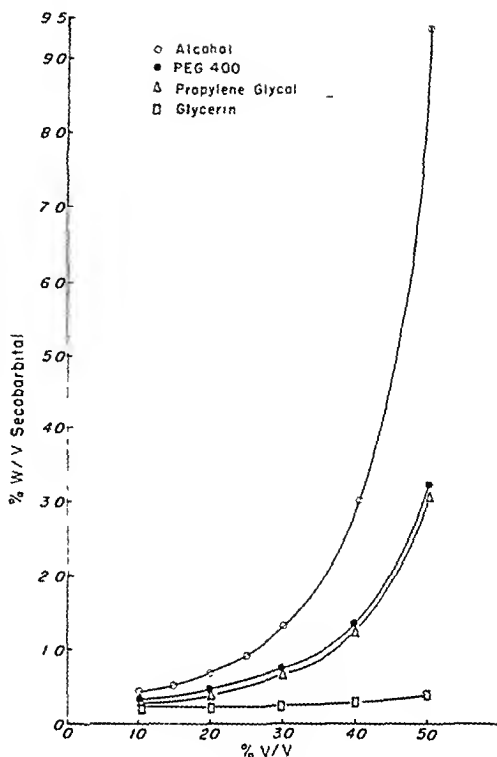


Fig. 2.—Solubility of secobarbital as function of solvent system and concentration.

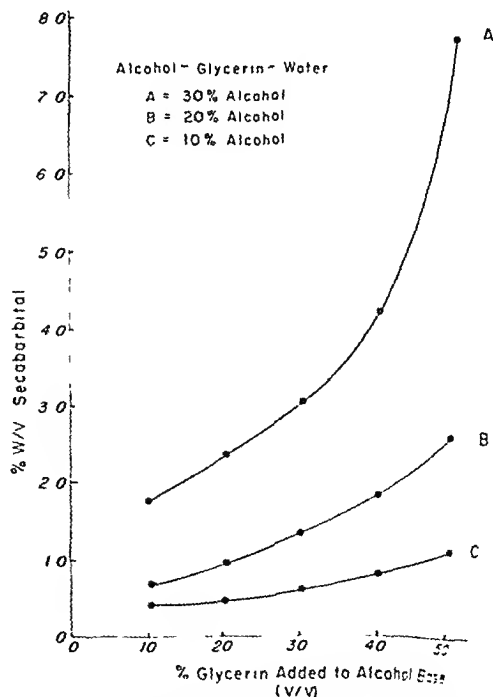


Fig. 3.—Solubility of secobarbital in a solvent system.

ent pH, and the flasks agitated for a period of twenty-four hours. In all instances, at each pH value, an excess of secobarbital was maintained.

At the end of the agitation period, the flasks were removed and the pH of the supernatant liquid determined. Each sample (supernatant liquid) was then acidified with 1.0 *N* hydrochloric acid to convert the salt into its acid form and extracted with chloroform in a manner described previously. The samples were then assayed for their drug content. For each pH value, duplicate samples were run. Table I includes the experimental results of solubility at the final pH. A solubility curve was drawn from the data in the table and is included in Fig. 4 (in water). In the same figure appears a theoretical solubility curve calculated from an equation developed by Higuchi (6)

$$S = (HA)(1 + K_a/H^+) \tag{Eq. 2}$$

where *S* is the total solubility of secobarbital, expressed in moles per liter, (*HA*) the acid solubility in the same units, *K*<sub>a</sub><sup>1</sup> the dissociation constant of secobarbital in water at 30°, and *H*<sup>+</sup> the hydrogen ion concentration. The calculated solubility values are included in Table I.

TABLE I.—SOLUBILITY OF SECOBARBITAL IN WATER AS A FUNCTION OF pH

Experimental		Theoretical <sup>a</sup>	
pH	Gm./100 ml.	pH	Gm./100 ml.
2.20	0.223	2.00	0.214
4.20	0.223	4.00	0.214
5.10	0.223	5.00	0.214
6.30	0.231	6.00	0.217
6.70	0.248	7.00	0.241
7.42	0.297	7.50	0.299
7.75	0.446	8.00	0.484
8.30	1.0750	8.50	1.069
8.50	1.653	9.00	2.916
8.55	1.943	9.40	7.002
8.90	4.591		

<sup>a</sup> Calculated from Eq. 2, using 1.26 × 10<sup>-4</sup> as *K*<sub>a</sub> and 9.0 × 10<sup>-3</sup> as (*HA*) concentration.

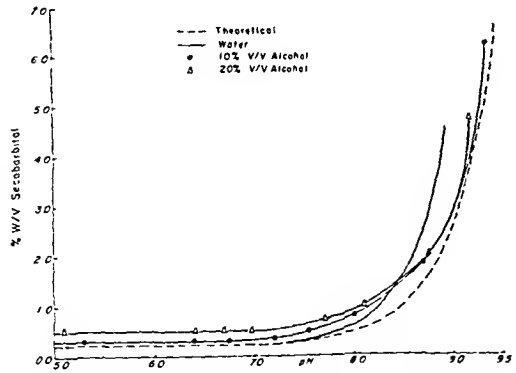


Fig. 4.—Solubility of secobarbital in alcohol-water systems as a function of pH.

Solubility as Function of Solvent and pH.—A series of solubility studies were conducted as described in the previous section except the binary

<sup>1</sup> *K*<sub>a</sub> is equal to 1.26 × 10<sup>-4</sup>.

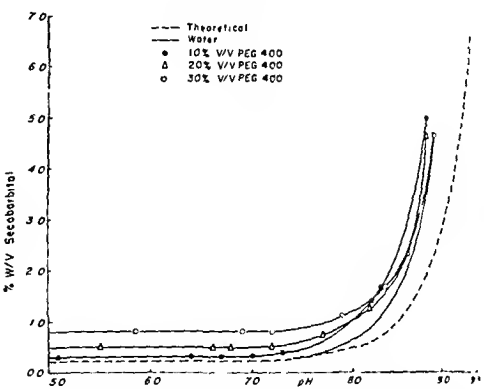


Fig. 5.—Solubility of secobarbital in PEG 400-water systems as a function of pH.

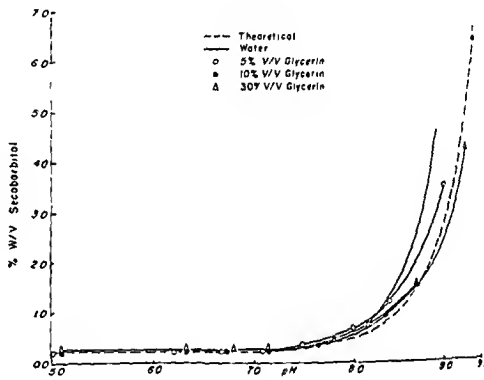


Fig. 6.—Solubility of secobarbital in glycerin-water systems as a function of pH.

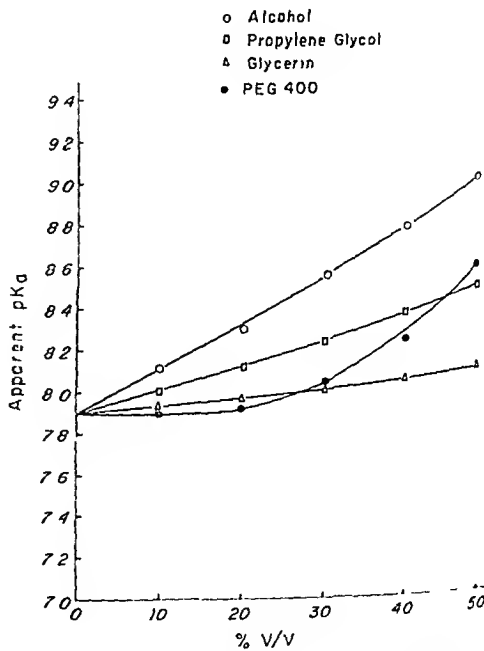


Fig. 7.—Effect of solvent on p*K*<sub>a</sub> of secobarbital

solvent systems were composed of various concentrations of either alcohol, glycerin, or polyethylene glycol 400 with water. The data obtained from these studies were plotted as shown in Figs. 4 to 6.

**Effect of Solvent upon Apparent  $pK_a$  Value.**—In order to determine the effect of the dissociation of secobarbital in various concentrations of alcohol, propylene glycol, glycerin, and polyethylene glycol 400, solutions were prepared containing a 1:1 salt to acid concentration. Each solution contained 0.26028 Gm. of secobarbital sodium to which was added exactly 5.00 ml. of a 0.1 *N* hydrochloric acid. The solutions were then adjusted to the proper per cent (v/v) of solvent system, placed into a water bath at 30° for four hours, and the pH of each solution was then determined. Since in a 1:1 buffer system the pH is equal to the  $pK_a$ , a plot was drawn in Fig. 7 relating  $pK_a$  to the various solvent concentrations.

## RESULTS AND DISCUSSION

**Temperature Effect.**—The linear relationship shown in Fig. 1 indicates that the solubility is related to the reciprocal of absolute temperature as predicted by van't Hoff's equation. Since the heat of solution ( $\Delta H$ ) is known, the solubility at any other temperature may be calculated by the use of another form of Eq. 1

$$\log \frac{S_2}{S_1} = \frac{\Delta H(T_2 - T_1)}{2.303 R (T_2 T_1)} \quad (\text{Eq. 3})$$

where  $S_1$  and  $S_2$  are the solubility in moles per 1,000 Gm. of solvent at absolute temperatures  $T_1$  and  $T_2$ , respectively.

The twenty-four-hour agitation period was found to be adequate to insure that equilibrium had been reached in the solution. Previous studies substantiated the absence of hydrolysis at the higher temperatures within the twenty-four-hour period for even those solutions having an alkaline pH.

**Solvent Effect.**—The phase diagram in Fig. 2 may conveniently be employed to find the exact solubility of secobarbital in concentrations (10–50% v/v) of alcohol, polyethylene glycol 400, propylene glycol, or glycerin at 30°. It will be noted that alcohol, in the higher concentration range, imparts the greatest degree of solubility. This is not too surprising, since, in general, a solvent system having a low dielectric constant should have greater dissolution activity to a relatively nonpolar compound such as secobarbital (undissociated). From the binary solvent systems included in the figure, alcohol-water has the lowest dielectric constant.

Polyethylene glycol 400 and propylene glycol dissolve approximately the same concentration of secobarbital in the 10–50% v/v concentration range of solvent. However, as may be seen in the figure, these two solvent systems have approximately one-half the solubilizing effect at 40% v/v concentration and one-third the effect at the 50% v/v concentration range, in comparison to alcohol.

In the solvent range studied, glycerin proved to be a rather poor solvent since even up to 50% v/v concentration, it had little ability to increase the solubility. Krause and Cross (7) found this to be true in their study on the solubility of phenobarbital.

Glycerin appears to have a synergistic solubilizing action upon alcohol-water systems. If Fig. 3 is stud-

ied closely and then compared to Fig. 2, it will be noted that in all instances glycerin increases the solubilizing effect over the binary system, alcohol-water. For example, to dissolve 5% w/v of secobarbital in 100 ml. of a solvent system, a 45% v/v alcohol-water system must be used, but this same quantity of secobarbital may be dissolved in an alcohol-glycerin-water system having volume concentrations of 30%, 45%, and 25%, respectively. Glycerin-water systems up to 50% barely solubilize 0.4% w/v of secobarbital.

**pH Effect.**—Even though the theoretical solubility curve for secobarbital was calculated using Eq. (2), the classical Henderson-Hasselbalch equation based upon the law of mass action may have been used.

$$\text{pH} = pK_a + \log \frac{(\text{Salt})}{(\text{Acid})} \quad (\text{Eq. 4})$$

However, this equation will express only the salt concentration, and at relatively high hydrogen ion concentration will not be useful since the concentration of salt to acid will be insignificant. In the case of secobarbital, the results by Eqs. 2 or 4 are practically identical above pH values of 7.0.

As may be seen by examining Fig. 4, the theoretical and experimental curves are in good agreement at the low pH values but diverge at the higher values. This finding is quite common when apparent dissociation constants and molar concentrations are used, rather than thermodynamic dissociation constants and activities. The equations, however, may be used to calculate approximate solubilities at a certain pH. Often this type of solubility calculation is very helpful to the product development group in formulation or in predicting incompatibilities.

**Solvent and pH Effect.**—Alcohol had a tendency to increase the solubility, as could be predicted, at the lower pH values and to decrease the solubility at the higher pH values. Polyethylene glycol 400 increased the solubility at all pH levels compared to the solubility of secobarbital in water. The excellent solvent and stabilizing action of the glycols for the barbiturates may be attested to by their inclusions in commercial barbiturate solutions. Glycerin decreased the solubility of secobarbital in the higher pH range. Unfortunately, time did not permit a more intensive investigation with higher concentrations of solvent as well as other solvent systems.

**Solvent Effect upon  $pK_a$ .**—It is well known that less polar solvents than water will suppress ionization of weak organic acids. This may be seen by referring to Fig. 7 where apparent  $pK_a$  values are plotted against volume concentrations of the solvents. All lines intersect at a  $pK_a$  value of 7.9, which is the  $pK_a$  value of secobarbital in water. The unilinear relationship with polyethylene glycol 400 is indicative that a molecular interaction between the solute and solvent has probably occurred.

## SUMMARY AND CONCLUSION

1. The solubility of secobarbital in water at various temperatures was determined and from this information the heat of solution was calculated. The heat of solution was found to be 4358.0 cal./deg./mole.



2. Binary solvent systems in which water was always the second solvent were found to increase the solubility in the order: glycerin-propylene glycol-PEG 400-alcohol.

3. Glycerin imparted a synergistic solubilizing activity in a three-component solvent system (alcohol-glycerin-water) upon the secobarbital.

4. Solubility curves as per cent w/v secobarbital *versus* pH were plotted from experimental data in water and in several mixed solvent systems.

5. The apparent pK<sub>a</sub> for secobarbital in various concentrations of alcohol, propylene glycol, glycerin, and polyethylene glycol 400 were

also determined and found to be linear in the range studied, except for polyethylene glycol 400.

6. Stability studies of secobarbital solutions are now in progress and will be presented in future papers.

## REFERENCES

- (1) Tomski, H. W., and Waller, L. J., *Pharm. J.*, 139, 421(1937).
- (2) Bailey, A. E., *ibid.*, 136, 620(1936).
- (3) Husa, W. J., and Jatul, B. B., *THIS JOURNAL*, 33, 217(1944).
- (4) Chatten, L. G., *J. Pharm. and Pharmacol.*, 8, 304(1956).
- (5) Mattson, L. N., *THIS JOURNAL*, 43, 22(1954).
- (6) Higuchi, T., Gupta, M., and Busse, L. W., *ibid.*, 42, 157(1953).
- (7) Krause, G. M., and Cross, J. M., *ibid.*, 40, 137(1951).

# Study of the Stability of Secobarbital Sodium Solutions II\*

## Separation and Identification of Degradation Products of Secobarbital Sodium

By A. J. KAPADIA and J. AUTIAN

A study was conducted to separate and identify degradation products of secobarbital sodium in an aqueous medium. Thermally degraded secobarbital sodium solutions were employed in the study. Extraction and paper chromatographic techniques were used to separate three degradation products of secobarbital sodium. Infrared analysis and chromatographic techniques identified the products as (a) mono ureide of 1-methylbutyl allyl malonic acid, (b) 1-methylbutyl allyl acetyl urea, and (c) urea. A fourth compound, 1-methylbutyl allyl acetic acid, was formed in the degradation but could not be isolated. The steps in the degradation of secobarbital sodium parallel those reported by Rotondaro for phenobarbital sodium.

SINCE THE TURN of the century, many reports have been published upon the accelerated decomposition of the various barbiturates in alkaline solutions. One of the first references to the decomposition of a barbituric acid derivative was found in a German patent issued in 1903 (1). The patent included information that diethylacetyl urea was formed on heating barbital. Husa and Jatul (2) reviewed the literature and presented a summary of the pathways of the decomposition of barbituric acid derivatives in an alkaline medium. Decomposition products of phenobarbital sodium have been isolated and identified by

Rotondaro (3). He identified the products as phenylethylacetyl urea, phenylethyl acetic acid, and urea. By paper chromatographic techniques Kapadia, Goyan, and Autian (4) effected a separation of the previously mentioned decomposition products of phenobarbital sodium.

Even though secobarbital has been in common medical use for some time, little published information is available concerning the products of degradation. This particular investigation was initiated to elucidate the decomposition products occurring in alkaline solutions of secobarbital and to compare the degradation steps to those of phenobarbital.

## EXPERIMENTAL

Supplies.—Whatman filter paper No. 1; secobarbital sodium U. S. P.; secobarbital; Urea, A. R.; *n*-hexyl alcohol, A. R.; ammonium hy-

\* Received August 21, 1959, from the College of Pharmacy, The University of Michigan, Ann Arbor.

Grateful acknowledgment is given to Dr. Harold Boaz, Eli Lilly and Co., Indianapolis, Ind., for the infrared and the titration data and their interpretation and to Mr. Ralph Huges for the microanalysis.

The authors wish to express their sincere appreciation to Eli Lilly and Co., for the generous supply of secobarbital.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

dioxide (28%) A. R.; borate buffer (pH 10) which consists of 43.9 ml. sodium hydroxide (0.1 *N*), 50.0 ml. boric acid (0.1 *M*), and purified water to make 100.0 ml.; potassium permanganate solution (0.02 *N*); *p*-dimethylaminobenzaldehyde solution (2% in 1.2 *N* HCl).

**Extraction Procedure.**—Secobarbital sodium solution (6% w/v) was prepared using purified water as the solvent and placed into ampuls. The ampuls were sealed and thermally degraded by storing at 80° for four days. After the storage period, several of the ampuls were broken and the solution filtered to recover the existing precipitate. The precipitate was washed and recrystallized several times from warm water. This compound was found to be neutral and had a melting point of 120°.<sup>1</sup>

The infrared spectrum of this compound is essentially identical with that of *n*-butyl allyl acetyl urea at the same concentration in chloroform except in the methyl and methylene CH stretching and bending regions where it differs slightly, but in the expected way for the disappearance of one CH<sub>2</sub> and the addition of one —CH<sub>3</sub>. As a reaction product of secobarbital this compound can be none other than 1-methylbutyl allyl acetyl urea (Fig. 1).

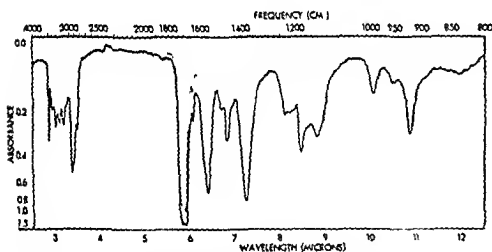


Fig. 1.—Infrared spectrum of 1-methylbutyl allyl acetyl urea in chloroform.

Approximately 20 ml. of the filtrate was placed into a 125-ml. separatory funnel and acidified with a few drops of dilute hydrochloric acid and extracted with 25 ml. of chloroform. This chloroform fraction was then transferred to another separatory funnel containing 25 ml. of a 0.1% sodium bicarbonate solution. The funnel was agitated slowly for several minutes and the organic phase removed. The remaining aqueous fraction was acidified with dilute hydrochloric acid and extracted with four portions of chloroform (20 ml.). These chloroform fractions were then pooled and the solvent evaporated. In order to obtain sufficient quantities of this compound, the above procedure was repeated a number of times, combining the resulting precipitates. Finally, the combined precipitate was recrystallized several times from methyl alcohol. The compound obtained was acidic in nature with a melting point of 140°.<sup>1</sup> The infrared spectrum, nitrogen analysis, and microtitration indicated that the compound was the mono ureide of 1-methylbutyl allyl malonic acid (Fig. 2).

**Paper Chromatographic Separation.**—An ascending paper chromatographic technique was employed for the separation of the several decomposition

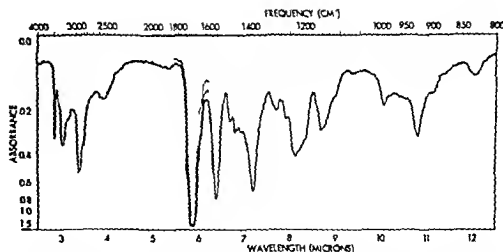


Fig. 2.—Infrared spectrum of the mono ureide of 1-methylbutyl allyl malonic acid in chloroform

products occurring in thermally degraded secobarbital sodium solution.

A two phase solvent system was employed in this study, consisting of the combination of the following solvents: *n*-hexyl alcohol, 200 ml.; borate buffer (pH 10), 25 ml.; ammonium hydroxide (28%), 12.5 ml.; purified water, 12.5 ml.

The above solvents were mixed in a separatory funnel for five minutes and then allowed to stand for two hours at room temperature, at which time the organic phase was removed. This phase was then used as the mobile solvent in the development of the chromatogram. The filter paper was sprayed with borate buffer (pH 10) and dried before use.

Aqueous solutions of secobarbital sodium and urea as well as chloroform solutions of 1-methylbutyl allyl urea and the mono ureide of 1-methylbutyl allyl malonic acid were spotted on the filter paper at equal intervals and at a starting point one and one-half inches from the bottom of the paper. These spots appear on the chromatogram (Fig. 3) at points A–D. At point E, spots were placed of the compounds mentioned above, superimposed upon each other. A spot of the aqueous fraction of the decomposed secobarbital sodium solution was placed at point F and a spot of the chloroform fraction at point G. A mixture of the aqueous and the chloroform (F and G) fractions were spotted at point H.

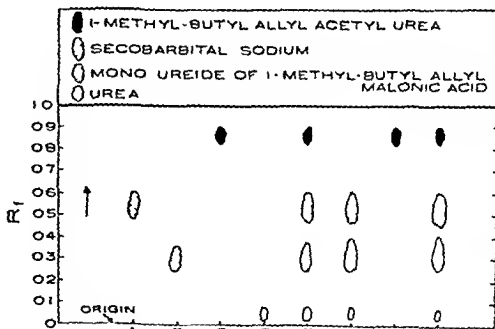


Fig. 3.—Separation of the decomposition products of secobarbital sodium by paper chromatography.

The paper was then placed into a battery jar containing a beaker of *n*-hexyl alcohol saturated with borate buffer-ammonium hydroxide-water solution. After an equilibration period, the mobile solvent system was added to the bottom of the jar. The chromatogram remained in the jar for a period

<sup>1</sup> Uncorrected temperature.

of eighteen hours, at which time it was removed and dried.

Three of the compounds (secobarbital, 1-methylbutyl allyl malonic acid, and 1-methylbutyl allyl acetyl urea) were viewed by spraying the chromatogram with 0.02 *N* potassium permanganate solution. For these compounds, a yellow spot on a red background was seen. The formation of these colors is indicative of compounds containing allyl groups. A bright yellow color developed for urea when the chromatogram was sprayed with *p*-dimethylamino-benzaldehyde solution.

The chromatographic technique made it possible to separate a number of the decomposition products from secobarbital sodium (Fig. 3). *R<sub>f</sub>* values of the various compounds are listed in Table I.

TABLE I.—*R<sub>f</sub>* VALUES FOR DECOMPOSITION PRODUCTS OF SECOBARBITAL SODIUM

Name of Compound	<i>R<sub>f</sub></i> Value <sup>a</sup>
Secobarbital sodium	0.58
Mono ureide of 1-methylbutyl allyl malonic acid	0.26
1-Methylbutyl allyl acetyl urea	0.88
Urea	0.09

<sup>a</sup> These values are the average of four determinations.

## DISCUSSION

It was assumed that alkaline degradation of secobarbital paralleled the steps reported by Rotondaro for phenobarbital in alkaline solutions. If this is the case then the more important degradation steps can be depicted as shown in Fig. 4.

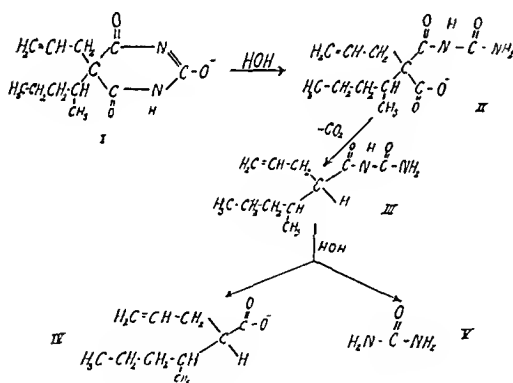


Fig 4.—Degradation steps of secobarbital in alkaline solution.

A hydrolytic cleavage occurs in the secobarbital (I) molecule producing the acidic compound (II), mono ureide of 1-methylbutyl allyl malonic acid, which decarboxylates to yield the neutral compound, 1-methylbutyl allyl acetyl urea (III). Further hydrolysis of this compound, gives 1-methylbutyl allyl acetic acid (IV) and urea (V).

It is interesting to note that neither Rotondaro (3) nor Kapadia (4) reported the isolation of the mono ureide of phenylethyl malonic acid (analogous to II) as a decomposition product of phenobarbital, even though Rotondaro includes the compound as a

decomposition product. In this particular study, compound IV could not be isolated by the techniques employed.

Since very little information was available as to actual decomposition products in secobarbital sodium solutions, it was first decided to isolate the several compounds by extraction methods. The thermal degradation accelerated the decomposition of the drug and produced a white precipitate as the first visible sign of decomposition. This precipitate was separated from the solution, purified, and subsequently identified as the neutral compound (III).

Acidulation of the alkaline solution and extraction with chloroform removed acidic and neutral compounds from the solution. Treatment of the chloroform fraction with bicarbonate solution separated the acidic decomposition product from secobarbital and any neutral product remaining. The pH of the bicarbonate solution was not sufficiently basic to convert the acid, secobarbital, to its salt form; thus, secobarbital remained in the chloroform phase. Finally, to isolate the acidic decomposition product from the aqueous phase from any neutral products, the solution was acidulated and the compound extracted with chloroform. On recovery of the compound and purification, the acidic compound was identified as the mono ureide of 1-methylbutyl allyl malonic acid (II).

With two of the decomposition products isolated and identified the paper chromatographic technique was developed to assist in rapid separation of the several degradation products occurring in secobarbital sodium solutions. This technique satisfactorily separated all the compounds shown in Fig. 4, but did not isolate compound IV. A number of methods of isolation were utilized to separate the compound but all proved fruitless.

## SUMMARY

1. A study was undertaken to isolate and identify the several decomposition products occurring in alkaline solutions of secobarbital.

2. By extraction procedure and infrared analysis it was possible to separate and identify one acidic compound (mono ureide of 1-methylbutyl allyl malonic acid) and one neutral compound (1-methylbutyl allyl acetyl urea).

3. Paper chromatographic techniques made it possible to separate the mono ureide of 1-methylbutyl allyl malonic acid, 1-methylbutyl allyl acetyl urea, and urea from decomposed secobarbital sodium solutions.

4. The results of the study indicate that the alkaline degradation of secobarbital solutions parallel those steps reported for phenobarbital.

## REFERENCES

- (1) German pat. 144,431, 1903. Von Niessen Brothers; through Bailey, A. E., *Pharm. J.*, 136, 620 (1936).
- (2) Hsiao, W. J., and Jatal, B. B., *This Journal*, 33, 217 (1914).
- (3) Rotondaro, F. A., *J. Assoc. Offic. Agr. Chemists*, 38, 809 (1955).
- (4) Kapadia, A. J., Goyan, J. E., and Autian, J., *This Journal*, 48, 407 (1959).

# The Hydrolysis of Hydrocortisone Phosphate in Essentially Neutral Solutions\*

By ARNOLD D. MARCUS

The degradation of hydrocortisone phosphate in essentially neutral solutions appears to involve hydrolysis as the only significant degradative pathway. The reaction is first order with respect to the steroid ester and seems linearly dependent upon the hydrogen ion concentration. The reaction is apparently an unusual 0.44 order with respect to the catalyst. The activation energy for the overall reaction has been found to be  $17.0 \pm 0.5$  Kcal./mole. The "frequency factor" of the Arrhenius equation has been calculated and an equation expressing the effect of both temperature and pH on the rate of the reaction is presented.

DURING THE FORMULATION of aqueous solutions of hydrocortisone phosphate it became apparent that the steroid ester was subject to both hydrolytic and oxidative degradation. Unfortunately, while oxidation could be minimized by adjusting the solutions to acid pH levels, the rate of hydrolysis increased markedly as the pH was lowered. Since, however, oxidation could be successfully controlled by the use of suitable antioxidants, the main problem concerned determination of the apparent minimum pH at which hydrolysis would proceed sufficiently slowly to permit adequate shelf lives for the formulated solutions.

Preliminary evidence, obtained by following the rate of formation of inorganic phosphate and/or the appearance of free hydrocortisone alcohol, indicated that the minimum suitable pH would be between 6 and 8. The rate of hydrolysis of hydrocortisone phosphate in this pH range was, therefore, studied with particular attention to the effect of temperature on the rate of reaction.

While the present study was by no means a comprehensive kinetic evaluation of the stability of hydrocortisone phosphate, it did provide the desired information in minimal time. It served, thereby, as an example of how chemical kinetic concepts and techniques permit most efficient use of accelerated stability testing programs.

## GENERAL DISCUSSION

Hydrocortisone phosphate is a dibasic acid,  $pK_1$  ca. 2 and  $pK_2 = 6.2$ . In the pH range 6 to 8, therefore, the ester could be presumed to be present both as the mono-anion and as the di-anion. Because of the negatively charged substrates, it appeared most likely that hydrolysis would be hydrogen ion-catalyzed. While hydroxyl ion-catalyzed hydrolysis could not be ruled out, the likelihood of electrostatic repulsion of a negatively charged catalyst appeared to minimize this latter

possibility. Indeed, other studies of the hydrolysis of structurally analogous phosphate ester (1, 3-5), showed precisely the behavior assumed above.

If it can be assumed that both anionic forms of hydrocortisone phosphate are subject to hydrogen ion catalyzed hydrolysis and that both reactions are first order with respect to each of the reactants, the general rate equation can be given as:

$$\frac{d(HCPO_4)}{dt} = k'H^+(H^+)(HCPO_4^-) + k''H^+(H^+)(HCPO_4^{2-}) \quad (\text{Eq. 1})$$

where  $(HCPO_4)$  is the total concentration of hydrocortisone phosphate,  $(HCPO_4^-)$  the concentration of the mono-anion and  $(HCPO_4^{2-})$  the concentration of the di-anion.  $k'H^+$  and  $k''H^+$  are the specific rate constants characteristic of the individual reactions.

Since the two anionic forms of the substrates should hydrolyze at different rates and inasmuch as the relative concentration of each species changes with variations in the pH of the solution, it appeared unlikely that there would be any simple, linear, relationship between the rate of reaction and the pH of the system.

## EXPERIMENTAL

**Reagents.**—Methylene chloride, Merck reagent, Porter-Silber reagent (2), 0.2 M phosphate buffers, pH 6-7.5.

**Method of Degradation.**—Exactly 2 Gm. of hydrocortisone phosphate, calculated as the free acid, was dissolved in a sufficient amount of each of the buffer solutions to yield exactly 200 ml. of solution. The solutions were sparged with nitrogen, subdivided into 5-ml. ampuls and sealed under nitrogen. The sealed ampuls were stored at 5° prior to starting the kinetic run.

For the kinetic runs, the ampuls were placed in a constant temperature bath set variously at 100, 91, 80, and  $70 \pm 0.1^\circ$ . When thermal equilibrium was attained one ampul representing each pH was removed, immediately chilled in an ice-water mixture, and stored at 5° until the contents were analyzed. These initial samples were arbitrarily designated the "zero-hour" samples. Other ampuls were removed at suitable intervals, chilled, and stored in the same manner.

**Analytical Procedure.**—Exactly 2 ml. of the solution in each ampul (at room temperature) was placed

\*Received August 21, 1959, from the Merck, Sharp & Dohme Research Laboratories, West Point, Pa.  
Presented to Scientific Section, A. P. A., Cincinnati meeting, August 1959.

in a separatory funnel along with approximately 25 ml. of distilled water and the mixture extracted with two 25-ml. portions of methylene chloride in order to remove any free alcohol. The aqueous phase was then drained into a 200-ml. volumetric flask and sufficient distilled water added through the separatory funnel to bring the solution in the flask to the mark.

Exactly 1-ml. aliquots of these extracted dilutions were then mixed with precisely 5 ml. of Porter-Silber reagent. The mixed solutions were placed in a constant temperature bath at 60° for two hours, after which the color intensity was determined against a distilled water-reagent blank at 410 m $\mu$ . Initially, all concentrations were calculated by comparison with a standard solution of hydrocortisone phosphate. Subsequently, the use of a standard was found unnecessary and all "concentrations" were obtained as a per cent of the "zero hour" sample.

## RESULTS AND DISCUSSION

**Order of the Reaction.**—From the plots shown in Fig. 1 it is evident that the hydrolysis of hydrocortisone phosphate is first order with respect to the steroid ester under a variety of experimental conditions. This is, of course, an expected result and is quite in keeping with the order found for the hydrolyses of other organic phosphate esters (1, 3-5). The observed first-order rate constants ( $k_{obs}$ ) are given in Table I.

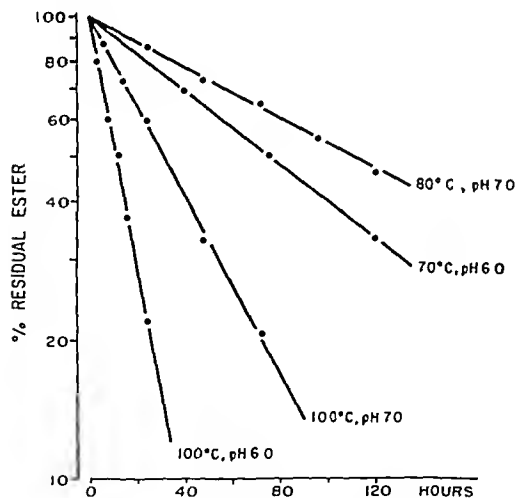


Fig. 1.—Plots showing the apparent hydrolysis of hydrocortisone phosphate to be first order with respect to the steroid ester under a variety of experimental conditions.

Unfortunately, the first-order disappearance rate found is not conclusive evidence that hydrolysis constituted the only degradative pathway. Definitive evidence towards this end could be obtained only by demonstrating the appearance of one mole of hydrocortisone alcohol for each molecule of the ester lost. Inasmuch as, under the conditions of this study, hydrocortisone alcohol degrades further into products which could not be detected

TABLE I.—MAGNITUDES OF THE OBSERVED PSEUDO FIRST-ORDER RATE CONSTANTS

T, ° C.	pH	$k_{obs}$ — hr. <sup>-1</sup> × 10 <sup>3</sup>
100	7.5	13.58
100	7.0	22.72
100	6.0	63.0
91	7.5	8.15
91	7.0	14.25
91	6.0	37.86
80	7.5	3.69
80	7.0	6.36
80	6.0	17.30
70	7.5	1.92
70	7.0	3.28
70	6.0	9.24

by either Porter-Silber color development or by ultraviolet absorption, this molecule-for-molecule relationship could not be demonstrated.

Despite this deficiency, the reproducibility of the observed rates and their strict adherence to first order kinetics are very strong evidence of hydrolytic rather than oxidative breakdown. This evidence is further strengthened by the preliminary evidence previously cited and by the extreme care taken to exclude oxygen from the reaction systems.

**pH Dependency.**—As noted earlier, it was assumed that the rate of hydrolysis would increase with increasing hydrogen ion concentration. As shown in Fig. 2, this is precisely the behavior exhibited by these systems. The plots in Fig. 2 run contrary to expectations, however, in that the reaction is linearly dependent on pH in a region within which a nonlinear response would, as discussed previously, be expected.

Under the conditions of the study, significant proportions of both anionic forms of the substrate could be expected to be present at each pH level. The linear relationship seems, therefore, merely fortuitous although it is interesting to note that a similar condition prevails in the hydrolysis of monobenzyl phosphate (1).

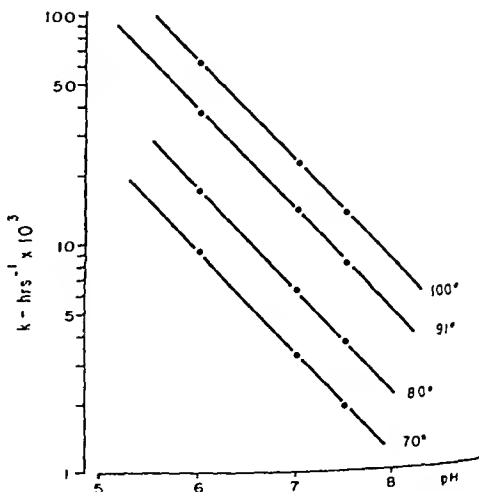


Fig. 2.—Plots indicating the effect of pH on the rate of hydrolysis of hydrocortisone phosphate. The order of reaction with respect to hydrogen ion, as determined from the slopes of these lines is 0.44.

This fortuitous linearity, while not unqualifiedly explicable at the present time, cannot be regarded as representing some simple, uncomplicated, reaction. If, for example, under the conditions of this study only one of the anionic forms of the substrate was present, the reaction would probably be second order, that is, first order with respect to each of the reactants. Since the reaction has already been demonstrated to be first order with respect to hydrocortisone phosphate and the observed rate constant is related to the apparent specific rate constant ( $k_{H+}$ ) by

$$k_{obs} = k_{H+} (H^+)^n \quad (\text{Eq. 2})$$

where  $n$  is the order of the reaction with respect to hydrogen ion, the slopes of the plots in Fig. 2 should be  $-1.0$  if the reaction was simply second order. The actual slope of these lines is, however,  $-0.44$  in each case. This is certainly not an apparent order representative of any simple reaction.

Instead, the apparent  $0.44$  order with respect to hydrogen ion probably represents a weighted average order of the individual reactions characterized by the observed rate constants and given by

$$k_{obs} = k'_{H+} (H^+)^{n'} + k''_{H+} (H^+)^{n''} \quad (\text{Eq. 3})$$

where specific rate constants are the same as given in Eq. 1 and where  $n'$  and  $n''$  are the orders with respect to hydrogen ion of the individual reactions

If it is true that the apparent  $0.44$  order represents a weighted average, the linear nature of the plots in Fig. 2 permits calculation of the apparent overall specific rate constant. Upon putting Eq. 2 in its logarithmic form we can write

$$\log k_{obs} = -n \text{ pH} + \log k_{H+} \quad (\text{Eq. 4})$$

and then

$$\log k_{obs} = -0.44 \text{ pH} + \log k_{H+} \quad (\text{Eq. 5})$$

thereby enabling us to calculate  $k_{H+}$  at each temperature. These calculated average values for  $k_{H+}$  are given in Table II.

**Temperature Dependency.**—The effect of temperature on the rate of hydrolysis of hydrocortisone phosphate is shown in the Arrhenius-type plots of Fig. 3. The activation energy as calculated from the slopes of these lines is  $17.0 \pm 1.0$  Kcal/mole. The same activation energy is obtained when the calculated average specific rate constants are plotted as a function of reciprocal absolute temperature.

Since it was desirable to write a kinetic equation expressing the effect of both pH and temperature on the rate of degradation, evaluation of the "frequency factor,"  $A$ , of the Arrhenius equation was necessary. This was calculated from the expression:

$$k_{H+} = A e^{-\Delta H_a/RT} \quad (\text{Eq. 6})$$

TABLE II—CALCULATED AVERAGE VALUES OF  $k_{H+}$  AT VARIOUS TEMPERATURES

$T, ^\circ \text{C.}$	$k_{H+}$
100	27.5
91	16.5
80	7.52
70	3.94

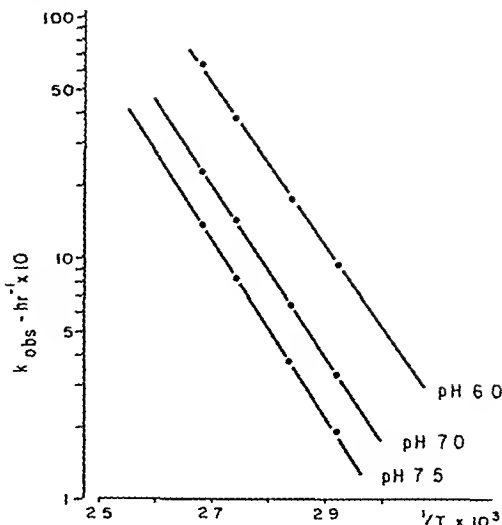


Fig. 3—Arrhenius type plots showing the temperature dependency of the hydrolysis of hydrocortisone phosphate. The apparent activation energy as determined from the slopes of these lines is  $17.0 \pm 1$  Kcal/mole.

and was determined to have an average value  $2.57 \times 10^{11}$ .

The value of  $A$  was calculated in terms of  $k_{H+}$  rather than  $k_{obs}$  because the former depends only on temperature while the latter is both temperature and pH dependent.

## KINETIC EQUATION

Since, from the Arrhenius equation we can write

$$\log k_{H+} = \log A - \Delta H_a/2.30 RT \quad (\text{Eq. 7})$$

and the values for  $A$ ,  $\Delta H_a$ ,  $R$ , and  $T$  are either known or have been evaluated, our equation becomes

$$\log k_{H+} = 11.41 - 17,000/4.58 T \quad (\text{Eq. 8})$$

When Eq. 6 is rearranged and substituted into Eq. 8, we get the kinetic equation

$$\log k_{obs} = 11.41 - 3710/T - 0.44 \text{ pH} \quad (\text{Eq. 9})$$

thus expressing the effect of temperature and pH on the pseudo first-order degradation of hydrocortisone phosphate.

In practice this equation has been applied with success to formulation procedures, stability evaluation, and in permitting separation of degradations resulting from both oxidation and hydrolysis.

Although the present communication considers only the pH range 6 to 7.5, the equation presented has proved reliable in describing degradation in the pH range 5.0 to 8.5.

## REFERENCES

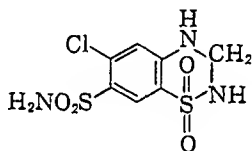
- (1) Kumamoto, J., and Westheimer, F., *J. Am. Chem. Soc.*, **77**, 2515 (1955).
- (2) Porter, C., and Silber, R., *J. Biol. Chem.*, **185**, 201 (1950).
- (3) Butcher, W., and Westheimer, F., *J. Am. Chem. Soc.*, **77**, 2420 (1955).
- (4) Chanley, J., and Feagenson, E., *ibid.*, **77**, 4002 (1955).
- (5) Chanley, J., et al., *ibid.*, **74**, 4347 (1952).

# The Photometric Determination of Hydrochlorothiazide and Its Hydrolysis Product\*

By C. R. REHM and J. B. SMITH

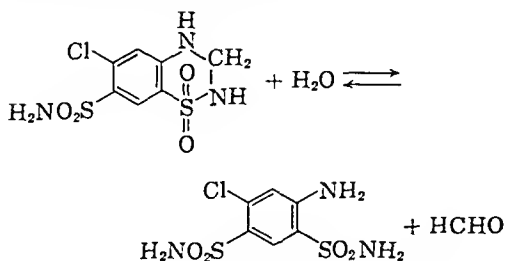
Photometric methods suitable for the routine determination of hydrochlorothiazide and its hydrolysis product, 4-amino-6-chloro-*m*-benzenedisulfonamide, have been developed. Hydrochlorothiazide undergoes an apparently reversible hydrolytic reaction in aqueous media to give 4-amino-6-chloro-*m*-benzenedisulfonamide and formaldehyde. Ultraviolet absorption measurements at 271  $m\mu$  are suitable for the determination of hydrochlorothiazide in the absence of the disulfonamide and for the determination of total hydrochlorothiazide (nonhydrolyzed plus hydrolyzed) where the extent of hydrolysis is less than 10 per cent. The disulfonamide resulting from the hydrolysis of hydrochlorothiazide is determined by reaction with nitrous acid and coupling of the diazonium compound formed with chromotropic acid. The resulting red color which is read at 500  $m\mu$  is linear in absorbance with concentration of disulfonamide. Hydrochlorothiazide and formaldehyde, as such, do not interfere, permitting the extent of degradation of hydrochlorothiazide to be accurately determined. In a similar manner, total hydrochlorothiazide is determined by alkaline saponification prior to diazotization and reaction with chromotropic acid. The intact hydrochlorothiazide is calculated from the difference between the results of the two colorimetric procedures

**H**YDROCHLOROTHIAZIDE<sup>1</sup> is an extremely active diuretic (1-5) and has the following molecular structure



It is a colorless crystalline compound with a melting point of 265-270° (decompn) and a molecular weight of 297.75. Hydrochlorothiazide is very slightly soluble in water, soluble in dilute sodium hydroxide solution and methanol, and very soluble in diethyleneglycol monomethylether.

In the course of developing analytical methods suitable for the determination of hydrochlorothiazide in pharmaceutical formulations, the degradation of this compound was also investigated. Studies in these laboratories have shown that in aqueous solution hydrochlorothiazide undergoes hydrolysis to give 4-amino-6-chloro-*m*-benzenedisulfonamide and formaldehyde according to the following reversible reaction



The instability of the  $\text{>N-CH}_2\text{-N<}$  group in a number of compounds yielding formaldehyde as hydrolysis product has been reported as early as 1907 by Votocek and Vessely (6). The hydrolysis of chlorothiazide in alkaline solution, which yields 4-amino-6-chloro-*m*-benzenedisulfonamide and presumably formic acid (salt), has been reported by Baer, *et al* (7). A study of the kinetics of the hydrolysis of hydrochlorothiazide, which will be presented in a subsequent paper, indicates that the equilibrium of the above reaction is far to the left.

In order to determine the stability of hydrochlorothiazide in solution and in various pharmaceutical formulations, specific analytical methods were required which would permit the extent of hydrolysis to be accurately determined. These methods are described in detail in the following sections.

## EXPERIMENTAL

**Apparatus and Reagents.**—Ammonium hydroxide 0.5 *N*, hydroxylamine HCl 0.7% solution, hydrochloric acid 1 *N*, sodium nitrite 1% solution, ammonium sulfamate 2% solution, chromotropic acid (disodium salt) 0.5% solution, freshly prepared, sodium acetate 1 *N*, hydrochlorothiazide standard solution, 0.1 mg/ml, 4-amino-6-chloro-*m*-benzenedisulfonamide standard solution, 0.1 mg/ml, 100° water bath, Beckman model B spectrophotometer or other suitable spectrophotometer.

**Determination of 4-amino-6-chloro-*m*-benzenedisulfonamide.**—If sample is a solid, dissolve accurately weighed sample in 5 ml of methanol or methyl cellosolve. Dilute sample with water to a volume such that final dilution contains 0.01 to 0.1 mg/ml of disulfonamide. Diluted sample should be assayed for disulfonamide within four hours if hydrochlorothiazide is present in the sample.

\* Received August 21, 1959 from the Research Department, Ciba Pharmaceutical Products Inc., Summit, N. J.  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 19, 1959.  
<sup>1</sup> Esdrix is the trademark of Ciba Pharmaceutical Products Inc., Summit, N. J.

to separate 50-ml. volumetric flasks, pipet 2 to 4 ml. of sample solution and 2 ml. of 4-amino-6-chloro-*m*-benzenedisulfonamide standard solution. To each flask add 1 ml. of sodium nitrite solution followed by 5 ml. of hydrochloric acid and mix well. Allow to stand for three to five minutes. Then add 1 ml. of ammonium sulfamate solution, mix well, and allow to stand for three minutes. To each flask add 1 ml. of chromotropic acid solution and 10 ml. of sodium acetate solution, dilute to volume with water, and mix well. Prepare a blank solution in a similar manner. Determine the absorbance of the sample and standard solutions against the blank solution in 1-cm. cells at 500  $m\mu$  in a suitable spectrophotometer.

**Calculation.**—Mg. disulfonamide =  $A_{\text{sample}}/A_{\text{standard}} \times 0.2 \times \text{dilution factor}$ ; mg. hydrolyzed hydrochlorothiazide = mg. disulfonamide  $\times 1.042$ .

**Determination of "Total" Hydrochlorothiazide.**—Dissolve or dilute sample with 0.5 *N* ammonium hydroxide solution so that a concentration of approximately 0.1 mg./ml. is obtained. Accurately pipet 2 ml. of sample solution and 2 ml. of standard hydrochlorothiazide solution into separate 50-ml. volumetric flasks, add 0.5 ml. of hydroxylamine HCl solution to each flask, and stopper tightly. Place the flasks in a water bath at 100° for sixty minutes. Cool the flasks to 25°, and proceed as directed under **Determination of 4-amino-6-chloro-*m*-benzenedisulfonamide** beginning with "to each flask add 1 ml. of sodium nitrite solution..."

**Calculation.**—Mg. "total" hydrochlorothiazide =  $A_{\text{sample}}/A_{\text{standard}} \times 0.2 \times \text{dilution factor}$ , where  $A_{\text{sample}}$  = absorbance of sample solution and  $A_{\text{standard}}$  = absorbance of standard solution; mg. hydrochlorothiazide (nonhydrolyzed) = mg. total hydrochlorothiazide<sup>2</sup> - mg. disulfonamide<sup>2</sup>  $\times 1.042$ .

## RESULTS AND DISCUSSION

**Ultraviolet Absorption Spectra.**—The ultraviolet absorption spectra of hydrochlorothiazide and of 4-amino-6-chloro-*m*-benzenedisulfonamide in methanol are shown in Fig. 1. Similar curves are obtained for aqueous solutions of the compounds. Absorbance measurements of methanolic and aqueous solutions of hydrochlorothiazide at the maximum of 271  $m\mu$  indicate that Beer's law is obeyed over the concentration range 1 to 10 mg./L. The  $E(1\%, 1 \text{ cm.})$  271  $m\mu$  of hydrochlorothiazide has been determined to be  $675 \pm 7$  (methanol) and  $650 \pm 7$  (water) in these laboratories.

It is evident from the overlapping spectral curves that ultraviolet absorption measurements are not suitable for the accurate determination of the hydrochlorothiazide moiety in the presence of the disulfonamide. Total hydrochlorothiazide (nonhydrolyzed + hydrolyzed) can be determined with fair accuracy by ultraviolet absorption measurements at 271  $m\mu$ , if the extent of hydrolysis (or contamination by the disulfonamide) is not too great. For example, if the disulfonamide were present to the extent of 10% of the amount of hydrochlorothiazide in a sample, a maximum error

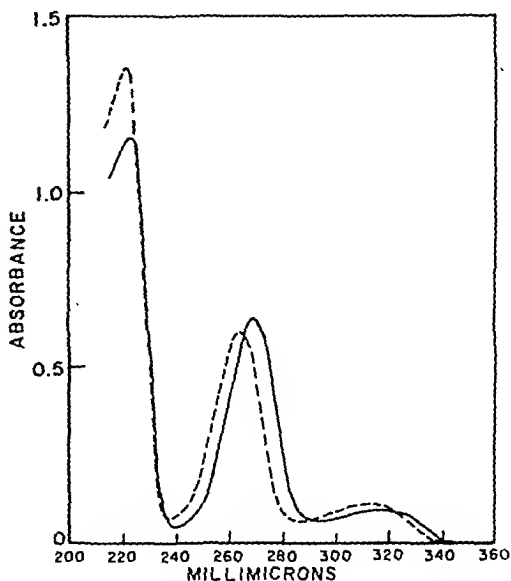


Fig. 1.—Ultraviolet spectra of hydrochlorothiazide and 4-amino-6-chloro-*m*-benzenedisulfonamide in methanol. — Hydrochlorothiazide, - - - - 4-amino-6-chloro-*m*-benzenedisulfonamide.

of approximately -1% would be introduced in the determination of the sum of the two compounds by absorbance measurements at 271  $m\mu$ .

**Color Reaction.**—The diazotization of 4-amino-6-chloro-*m*-benzenedisulfonamide and subsequent coupling with *N*-(1-naphthyl)-ethylenediamine by the well-known Bratton and Marshall procedure has been used by Baer, *et al.* (7), in the determination of chlorothiazide with reportedly excellent results. Chromotropic acid (4,5-dihydroxy-2,7-naphthalenedisulfonic acid) which has been reported to give highly colored products upon reaction with diazotized *o*-aminobenzenesulfonamides (8) was used as the coupling agent in these procedures.

The diazotization reaction is complete in two minutes or less as shown by potentiometric titrations with sodium nitrite. The titrations also revealed that 2 moles of nitrous acid are consumed by one mole of the compound, indicating the reaction is apparently more complex than a simple diazotization reaction. The reaction between the diazonium compound and chromotropic acid after destruction of the excess nitrous acid results in a pink color with an absorption maximum at 500  $m\mu$ . The color development proceeds somewhat slowly in acid medium, requiring thirty to forty-five minutes for full color development. By buffering the solution to a higher pH (approximately 4.7) through the addition of sodium acetate, the color development is very rapid and absorbance values may be determined within five minutes. Absorbance values have been found to be constant over a period of several hours, although the solutions gradually attain an orange tint, apparently due to slow oxidation of the excess chromotropic acid.

The buffering of the final solution accounts, in part, for the reproducible absorbance values obtained with the standards. The relationship between absorbance and concentration is linear

<sup>2</sup> Values computed on basis of same total initial sample weight or volume.



over a wide range of concentration as shown in Fig 2. The  $E(1\%, 1\text{ cm})$  at  $500\text{ m}\mu$  in terms of original disulfonamide concentration is approximately 1,080.

In order to determine the suitability of the method for kinetic and stability studies, known samples of the disulfonamide in the presence of hydrochlorothiazide and formaldehyde were analyzed. These data which are shown in Table I indicate that the compound can be accurately determined in the presence of large molar excess amounts of hydrochlorothiazide and formaldehyde. Hydrochlorothiazide is sufficiently stable in dilute acid solution at room temperature so that no significant hydrolysis occurs during the diazotization procedure. Formaldehyde which forms an intense purple color with chromotropic acid in 80% sulfuric acid (9) apparently does not react at room temperature in acid solutions which are less than 2 *N*.

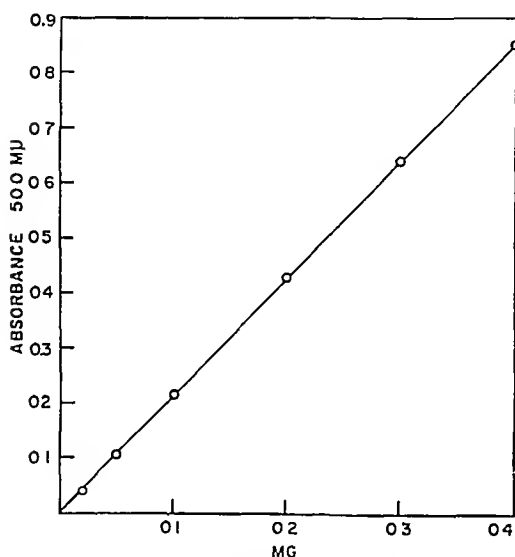


Fig 2—Plot of absorbance vs concentration of 4-amino-6-chloro-*m*-benzenedisulfonamide after diazotization and coupling with chromotropic acid. Final volume = 50 ml.

TABLE I—DETERMINATION OF 4-AMINO-6-CHLORO-*m*-BENZENEDISULFONAMIDE IN PRESENCE OF HYDROCHLOROTHIAZIDE AND FORMALDEHYDE\*

Sample Composition, mg			Disulfonamide Found, mg
Disulfonamide	Hydrochlorothiazide	Formaldehyde	
1.00	0	..	1.01
1.00	0.50	..	1.00
1.00	1.00	..	0.99
1.00	10.00	..	1.00
1.00	50	..	1.01
1.00	..	0.01	1.00
1.00	..	0.10	1.00
1.00	..	1.00	0.99
1.00	1.00	0.10	0.99

\* Results are averages using duplicate samples and standards.

**Hydrolysis Reaction.**—Studies of the decomposition of hydrochlorothiazide in aqueous solution have indicated that the hydrolysis proceeds to an apparent state of equilibrium, the extent of hydrolysis being dependent upon the concentration of drug in solution. Typical results obtained for the hydrolysis in sodium hydroxide and in ammonium hydroxide solution, as shown in Fig 3, indicate that hydrolysis is incomplete even after ninety minutes. Refluxing in 2 *N* sodium hydroxide for sixty minutes effected complete hydrolysis, presumably by destroying the formaldehyde released, thus forcing the reaction to completion. The procedure which would be inconvenient for routine determinations did suggest, however, that the reaction could be forced to completion under milder conditions by the addition of a suitable agent to react with the formaldehyde as it was formed.

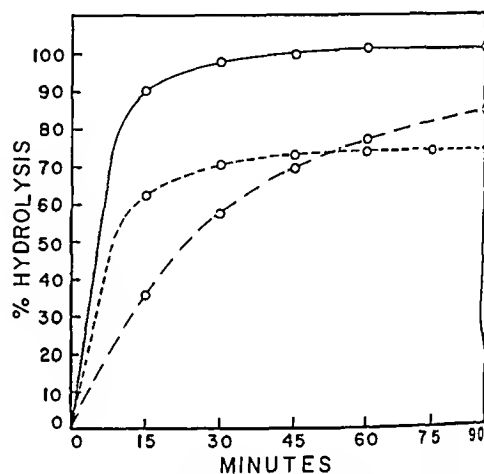


Fig 3—Hydrolysis of hydrochlorothiazide in alkaline media: — 0.5 *N* NH<sub>4</sub>OH + hydroxylamine HCl, - - - 0.5 *N* NH<sub>4</sub>OH, ···· 1 *N* NaOH.

The addition of a small amount of hydroxylamine HCl to solutions of hydrochlorothiazide in 0.5 *N* ammonium hydroxide resulted in complete hydrolysis in approximately forty-five minutes at 100°, as shown in Fig 3. A reaction time of sixty minutes was found to be suitable. Although hydroxylamine undergoes hydrolysis in alkaline solution, it is apparently sufficiently stable in ammonium hydroxide at 100° as to be available for reaction with the formaldehyde resulting from the hydrolysis of hydrochlorothiazide. Sufficient nitrite must be added in the subsequent diazotization reaction to provide an excess over that consumed by the hydroxylamine remaining.

Although analyses of hydrochlorothiazide using the disulfonamide as a standard have shown that the hydrolysis is essentially 100% over a concentration range of 0.01 to 0.1 mg/ml, it is preferable to use hydrochlorothiazide standards, carrying them through the same procedure as unknown samples.

Known samples of hydrochlorothiazide plus 4-amino-6-chloro-*m*-benzenedisulfonamide, representing hydrochlorothiazide hydrolyzed to different extents, as shown in Table II, were analyzed for

TABLE 11.—DETERMINATION OF HYDROCHLOROTHIAZIDE AND 4-AMINO-6-CHLORO-*m*-BENZENEDISULFONAMIDE IN MIXTURES

Sample Composition, mg <sup>a</sup>		Found, mg <sup>a</sup>		% Hydrolysis	
A	B	B	C	Theory	Experimental
100.0	0	0 05	101	0	0 05
95 0	4 80	4 84	100	5	5 05
90 0	9 60	9 69	100	10	10 2
75 0	24 0	24 3	99	25	25 5
50 0	48 0	48 0	100	50	50 0
25 0	72 0	71 5	100	75	74 5
10 0	86.4	87 3	100	90	91

<sup>a</sup> A = hydrochlorothiazide, B = 4-amino-6 chloro-*m*-benzenedisulfonamide, A + B corresponds to 100 mg of A hydrolyzed to extent shown, C = total of A + B determined as A, % hydrolysis (experimental) = (B × 1.042/C) × 100

total hydrochlorothiazide and for the disulfonamide content by the procedures outlined. The per cent hydrolysis was calculated from the difference between the assay for total hydrochlorothiazide and the assay for the disulfonamide for each sample. It is necessary, of course, to convert the determined disulfonamide content of the sample to an equi-

valent amount of hydrochlorothiazide by multiplying by the factor 1.042, the ratio of the molecular weights. From the data shown it is apparent that hydrochlorothiazide can be determined with a precision of ±1% and that the extent of hydrolysis can be accurately determined. These procedures are applicable to the determination of hydrochlorothiazide and its stability in tablets and liquid formulations and have been used in these laboratories on a routine basis.

## REFERENCES

(1) Herrmann, G. R., Hejtmancik, M. R., and Kroetz, F. W., *Texas State J. Med.*, **54**, 854 (1958)  
(2) de Stevens, G., Werner, L. H., Halamandaris, A. and Ricca, S., Jr., *Experientia*, **14**, 463 (1958)  
(3) Brest, A. N., and Likoff, W., *Am. J. Cardiol.*, **3**, 144 (1959)  
(4) Goldberger, E., *ibid.*, **3**, 14 (1959)  
(5) R. A., Sheppard, H., and V., *ibid.*, **18**, 366 (1959)  
(6) V., *ibid.*, **40**, 410 (1907)  
(7) Baer, J. E., Leidy, H. L., Brooks, A. V., and Beyer, K. H., *J. Pharmacol. Exptl. Therap.*, **125**, 295 (1959)  
(8) Fierz, H. E., Schlittler, E., and Wakmann, H., *Helv. Chim. Acta*, **12**, 663 (1929)  
(9) Eegriwe, E., *Z. anal. Chem.*, **110**, 225 (1937)

# The Metabolism and Central Nervous System Distribution of C-14 Carbonyl Salicylamide in the Rat\*

By WILLIAM F. BOUSQUET and JOHN E. CHRISTIAN

This investigation was undertaken in order to define the central nervous system distribution pattern of C-14 carbonyl salicylamide in the rat, as a possible basis of explaining the higher level of analgetic activity of this drug as compared to other compounds of the salicylate group. The metabolic products of salicylamide in this species were identified. Various tissues were then examined for the presence of metabolic products.

NUMEROUS STUDIES (1-3) have shown salicylamide to possess favorable analgetic properties. Blood levels of this compound are low in man and animals (4), presumably due to rapid distribution to the tissues (5). In experimental animals brain concentrations of salicylamide are higher than other salicylate drugs (6).

The uptake of C-14 carbonyl salicylamide by the central nervous system of the rat has been

studied at various time intervals following intraperitoneal administration of 100 mg./Kg of salicylamide containing 3.0  $\mu$ c of C-14 activity. Samples were taken from the blood, cerebrospinal fluid, cerebral cortex, cerebellum, medulla, pituitary, hypothalamic area, and the adrenal glands at intervals of five, fifteen, thirty, sixty, and one hundred and twenty minutes after drug administration.

Various metabolic products of salicylamide have been reported in studies involving both animals and man. The predominant product observed has been salicylamide itself, either in the free state or conjugated with either sulfate or glucuronate. Studies of salicylamide metabolism in humans have failed to reveal the presence of the 5-hydroxy analog of salicylamide (gentisamide) as a metabolite (7), while this compound has definitely been established as a metabolic product in animals (8).

\* Received August 21, 1959, from the Bionucleonics Department, Purdue University, Lafayette, Ind.  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

Other authors (9) have reported the presence of gentisic acid in the pituitary gland of febrile rats following salicylic acid administration. Accordingly, it was of interest in this work to determine whether the pituitary plays a role in the hydroxylation mechanism leading to the formation of gentisamide from salicylamide.

The metabolic products present in the urine of both normal and hypophysectomized animals following administration of 100 mg./Kg. of salicylamide containing 3.0  $\mu$ c. of C-14 activity were isolated and identified. The blood, whole brain, pituitary, cerebrospinal fluid, and the adrenal glands were examined in another group of animals in order to determine if these areas contained any metabolic products.

### EXPERIMENTAL

**Synthesis of C-14 Carbonyl Salicylamide.**—This compound was prepared by a modification of the method of Bousquet and Christian (10), using C-14 carboxyl salicylic acid synthesized in these laboratories by Bousquet and Christian (11). The compound was determined to be both chemically and radiochemically pure before use in the animal experiments. This material was found to have a specific activity of  $4.05 \times 10^6$  dpm/mg.

**Central Nervous System Distribution Studies.**—Male, albino rats of the Holtzman strain were used throughout this work. These animals were maintained on Purina Laboratory Chow. At the time of use the animals weighed between 200 and 310 Gm. They were not fasted prior to injection.

**Procedure.**—Five animals were studied at each time interval following intraperitoneal administration of 100 mg./Kg. of salicylamide containing 3.0  $\mu$ c. of C-14 activity. The drug was dissolved in 40% aqueous propylene glycol with gentle heating. The volume of solution administered to each animal was 1.0 ml. Animals were sacrificed at five, fifteen, thirty, sixty, and one hundred and twenty minutes after drug administration.

All radioactivity determinations were made with the Tri-Carb liquid scintillation spectrometer.<sup>1</sup> Special, low potassium content glass vials<sup>2</sup> were used as sample containers. Tissue samples were dissolved in 2 ml. of a 1 M solution of Hyamine<sup>3</sup> hydroxide in methanol. Correction for differences in sample counting efficiency was made using an internal standard technique (12).

After injection the animals were placed in individual cages. One minute before expiration of the time period allowed for uptake of the drug, the animal was removed from the cage, anesthetized lightly with ether, and a sample of cerebrospinal fluid withdrawn by puncture of the cisterna magna with a 27-gauge needle fitted with a length of polyethylene tubing. The fluid was allowed to flow to a predetermined height in the tubing such

that about 40 mg. was obtained. In some cases it was necessary to apply suction to the tubing. The fluid sample was transferred to a sample vial containing 2.0 ml. of Hyamine solution, which was capped and set aside.

The animal was then decapitated and the free flowing blood collected in a three-inch evaporating dish containing 10 drops of a saturated solution of ammonium oxalate. This was transferred to a 15-ml. tube and centrifuged for twenty minutes. Three 25- $\mu$  plasma samples were transferred to sample vials containing Hyamine as described for the CSF samples.

The brain was perfused through the carotid using 15 ml. of 0.85% saline. The skull was opened, the brain removed intact, rinsed rapidly with saline, and set aside on a piece of paper toweling moistened with saline. The pituitary was then removed and treated likewise. The adrenal gland were removed and dissected free of fat.

The pituitary and the adrenals were weighed and transferred to sample vials containing 2.0 ml. of Hyamine. Two samples of the medulla, cerebellum, cerebral cortex (no white matter), and the hypothalamic area from each animal were dissected out, weighed, and transferred to sample vials. All tissue samples taken were less than 40 mg. in weight.

When all tissue samples from a given group of animals had been taken, they were set aside in the sample vials to allow dissolution to begin. After twenty-four hours all samples with the exception of the plasma and CSF were placed in an oven and heated at 60° until the tissue dissolved. This usually required not over seventy-five minutes if the caps were removed from the vials prior to heating. It is imperative that the temperature not exceed 60°. Higher temperatures cause the solutions to become dark brown in color and counting efficiency is greatly impaired. Upon completion of tissue dissolution the vials were removed from the oven, cooled to room temperature, 15 ml. of liquid phosphor solution<sup>4</sup> added, and the samples counted in the Tri-Carb.

**Results.**—The results obtained in the central nervous system distribution study are shown graphically in Fig. 1. Activities are expressed on a wet weight basis. Maximal central nervous system uptake of C-14 carbonyl salicylamide in the rat is seen to occur between five and fifteen minutes following administration of the labeled compound. No well-defined peak is seen for the blood, but higher concentrations are evident between thirty and sixty minutes. Between sixty and one hundred and twenty minutes there appears to be little change in concentration in any area but the hypothalamus.

The concentration ratio between the cerebrospinal fluid and the plasma reached unity in twelve minutes, indicating rapid transport from the plasma. It is of interest that salicylic acid, a substance of similar molecular structure, requires over six hours to reach equivalent concentration in the cerebrospinal fluid of rabbits (13).

<sup>1</sup> Packard Instrument Co., LaGrange, Ill.

<sup>2</sup> Five-dram, crystallite vial, FA-3305, T. C. Wheaton Co., Millville, N. J.

<sup>3</sup> A high molecular weight quaternary ammonium compound which dissolves animal tissue. Supplied by the Rohm and Haas Co. It was converted from the hydrochloride to the free base by treatment with sodium hydroxide.

<sup>4</sup> Prepared by dissolving 102 Gm. of naphthalene, 6.5 Gm. of PPO, 2.5 Gm. of POPOP, and 0.13 Gm. of POPOP (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100) in a mixture of xylene and absolute ethanol, 300 ml.

The degree of separation achievable between the suspected metabolic products of salicylamide using these solvent pairs is shown in Table I. In deter-

TABLE I.— $R_f$  VALUES OF POSSIBLE SALICYLAMIDE METABOLITES

Compound <sup>a</sup>	$R_f \times 100$	
	Solvent A <sup>b</sup>	Solvent B <sup>c</sup>
Salicylic acid	51	95
Salicylamide	88	95
Gentisic acid	23	23
Gentisic acid amide	86	14

<sup>a</sup> Ten micrograms of each compound used Running times for a 20-cm rise were approximately eight hours for solvent A and three hours for solvent B at room temperature

<sup>b</sup> Isopropanol:water, 100:10.

<sup>c</sup> Benzene:glacial acetic acid, 80:20

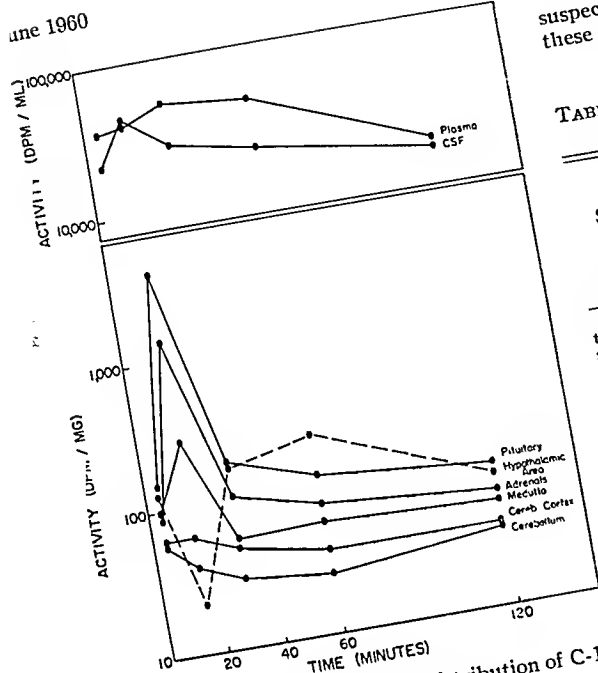


Fig. 1.—Central nervous system distribution of C-14 carbonyl salicylamide in the rat.

The low value obtained for the fifteen-minute sample from the hypothalamic area was checked using data from three additional animals. The results obtained (21 dpm vs. 23 dpm) indicate that this low value was not due to error in sample preparation.

**Metabolism of C-14 Carbonyl Salicylamide.—**  
**Procedure.**—The animals used in this study were not fasted prior to use and were watered but not fed during the time of urine collection. Administration of 100 mg./Kg. of salicylamide containing 30  $\mu$ c. of C-14 activity was made by intraperitoneal injection. Seven animals were studied.

Immediately after injection, each animal was placed in a glass metabolism chamber similar to that described by Roth, *et al.* (14), the urine being collected separately from the feces over a twenty-four hour period. The collection flask contained 1.0 ml. of glacial acetic acid to prevent alkaline oxidation of any diphenolic metabolites formed.

At the end of the collection period, the urine was filtered and an aliquot taken for radioactivity analysis. The remainder of the urine was acidified with concentrated sulfuric acid and immediately extracted with three volumes of ether in three divided portions. The ether extract was reduced in volume by evaporation so that 10  $\lambda$  of whole was equivalent to approximately 100  $\lambda$  of urine. An aliquot of this extract was taken for radioactivity analysis by two-dimensional paper partition chromatography. Solvent pairs employed were isopropanol:water, 100:10 (solvent A); and benzene:glacial acetic acid, 8:2 (solvent B). Whatman No. 1 paper was used. After development the papers were examined under ultraviolet light to locate gentisates, and then sprayed with ferric nitrate solution to locate salicylates. Another aliquot of this extract was taken for radioactivity analysis

mining the fluorescence of gentisates it is important to dry the papers thoroughly until the odor of acetic acid is no longer perceptible.

The acidified urine remaining was transferred to a 50-ml. round-bottom flask fitted with a reflux condenser and heated in a boiling water bath for twenty-five minutes to hydrolyze any conjugates present. Upon cooling to room temperature, this was then extracted with ether and concentrated as described above. Samples of this extract were both chromatographed and analyzed for radioactivity.

A control was run wherein the suspected metabolic products of salicylamide containing the amide function (salicylamide and gentisic acid amide) were added to urine, the urine then hydrolyzed, extracted, and chromatographed as above. No breakdown to salicylic or gentisic acids was detected under the hydrolysis conditions employed. Another control urine was run and carried through the hydrolysis and extraction procedures. Upon chromatography of this urinary extract, no materials were present which fluoresced under ultraviolet light<sup>5</sup> or gave a color reaction with ferric nitrate solution.

The experiments were repeated using hypophysectomized animals<sup>6</sup> in order to determine if the pituitary plays a role in the hydroxylation mechanism. These animals were maintained on a diet consisting of: Canned dog food, 480 Gm.; skim milk, 480 ml.; and 2 slices of crumbled whole wheat bread, the ingredients being well mixed. Five per cent dextrose was supplied as drinking water (15). Administration of the drug, collection, and treatment of urine samples were identical to that described for the normal animals, except that the urine was immediately hydrolyzed as it was of interest only to determine if any gentisic acid amide was present.

**Results.**—Radioactivity determinations on the whole urine of both the normal and hypophysectomized animals indicated that about 96% (range 91-99%) of the administered radioactivity was present in the urine after twenty-four hours. Radioactivity present in the ether extract of the unhydrolyzed urine from normal animals showed

<sup>5</sup> Blak-Ray long wave ultraviolet light, model B-50, Ultra-Violet Products Inc., South Pasadena, Calif.  
<sup>6</sup> Sprague-Dawley strain, Male, 250 Gm. Supplied by the Hormone Assay Laboratories, Chicago, Ill.

that about 3% (range 1.7–7.4%) of the compound was excreted in the free form, assuming that extraction was complete and that only free salicylamide was extracted.

Chromatographic analysis of the ether extract of the unhydrolyzed urine from normal animals revealed the presence of only one spot corresponding to free salicylamide. In two animals, an additional spot was present upon spraying with ferric nitrate. This was probably salicyluric acid, although a definite identification was not made. No gentisic acid amide was found in these extracts indicating that any of this compound formed *in vivo* is not excreted in the free form.

Chromatography of the ether extracts of the acid hydrolyzed urines showed the presence of two spots, one corresponding to salicylamide and one to gentisic acid amide. These were observed in both normal and hypophysectomized animals, thus suggesting that the pituitary does not play a role in the hydroxylation mechanism. In order to confirm the presence of gentisic acid amide in the extracts this compound was added to the extract and chromatographed again. A single fluorescent spot was present which was found to contain the radioactivity.

It was of interest to determine the relative amounts of salicylamide and gentisic acid amide present in the urine after hydrolysis. Accordingly, the chromatogram spots containing these materials were cut from the paper and analyzed for their radioactivity. These data indicate that between 5 and 30% of the excreted radioactivity is associated with gentisic acid amide.

It was not possible to show the presence of either salicylic acid or gentisic acid in the urinary extracts. Thus it appears that the amide function is not broken down during salicylamide metabolism in this species. The biostability of the amide function is well recognized (16, 17).

**Tissue Metabolites of C-14 Carbonyl Salicylamide.**—It was of interest to determine if the hydroxylated metabolite of salicylamide which presumably (18, 19) is formed in the liver of experimental animals, is returned to the general circulation and distributed to the body tissues. The plasma, cerebrospinal fluid, pituitary gland, adrenal glands, and the brain were examined for the presence of metabolites.

**Procedure.**—Male rats were dosed as previously described. Fifteen minutes after drug administration a sample of cerebrospinal fluid was withdrawn from each anesthetized animal. A 10- $\lambda$  sample of CSF was chromatographed using the solvent systems described for the urine samples.

At twenty minutes the animals were sacrificed by decapitation, the blood being collected with oxalate. The pooled blood from three animals was centrifuged and 4.0 ml. of clear plasma removed with a pipet. To this was added 2.0 ml. of a 10% solution of trichloroacetic acid and the sample again centrifuged. Four milliliters of clear liquid was withdrawn. This was extracted with three 10-ml. portions of ether and concentrated so that 20  $\lambda$  of extract was equivalent to approximately 100  $\lambda$  of plasma. Twenty- $\lambda$  portions of the extract were chromatographed.

The brains were pooled and homogenized with 7

ml. of saline. The total volume of homogenate was about 13 ml. One milliliter of concentrated hydrochloric acid was added and the homogenate extracted with five 25-ml. portions of ether. The extract was concentrated to 1 ml., and 15- $\lambda$  portions chromatographed.

The pituitaries from the three animals were transferred to a test tube containing 1.0 ml. of 500 mg. % solution of pepsin in 0.1 *N* hydrochloric acid. This was heated at 38° for eighteen hours. The fine suspension produced was extracted with three 10-ml. portions of ether, the extract concentrated to 100  $\lambda$ , and 15- $\lambda$  aliquots chromatographed.

The adrenals were treated in the same manner as the pituitary glands.

**Results.**—Examination of the chromatograms of the tissue extracts revealed the presence only of salicylamide. This was found in all of the extracts. Under ultraviolet light no fluorescence was found in the area where gentisic acid amide would be present. In order to confirm the finding that gentisic acid amide is not present in the tissue following salicylamide administration, the chromatograms of the tissue extracts were exposed to Eastman-Kodak "No-Screen" X-ray film for a period of one month. This technique would serve to indicate the presence of minute amounts of gentisic acid amide which would not be detectable by conventional procedures. At the end of the exposure period no darkening of the film was observed.

## DISCUSSION

The finding of highest activity in the pituitary gland following administration of C-14 carbonyl labeled salicylamide is in agreement with reports of other authors who found the same to be true in the case of labeled aspirin (20). Further, these data support an earlier report of Bousquet and Christia (10) that whole brain concentrations of salicylamide are much higher than plasma concentrations.

The greatest positive slope of the plasma curve coincides in time with the greatest negative slope of the tissue curves. Assuming that complete absorption had taken place, this would indicate that peak plasma levels cannot be reached until the compound is removed from the tissues.

The finding of highest activity in the pituitary cannot readily be explained. Although the pituitary is thought to lie outside the blood-brain barrier, the possibility exists that this area selectively concentrates the drug. The high uptake of radioactivity by the pituitary warrants further study regarding the mechanism of action of antipyretic-analgesic drugs of this type. Other authors (9) report that gentisic acid is present in the pituitary gland of febrile rats following administration of aspirin or salicylic acid. It should be emphasized that the animals used in this study were not in the febrile state. However, the finding of identical metabolites in normal and hypophysectomized animals would seem to rule out any normal hydroxylating function of this gland. The possibility does exist that the disposition of hydroxylated metabolites would be different in febrile animals.

## SUMMARY AND CONCLUSIONS

C-14 carbonyl labeled salicylamide was synthesized from C-14 carboxyl salicylic acid by a modification of the method of Bousquet and Christian. Chemical and radiochemical purities of the labeled compound were determined by paper partition chromatography and autoradiography.

The distribution of C-14 carbonyl salicylamide in the central nervous system, adrenals, and plasma of the rat was studied at time intervals of five, fifteen, thirty, sixty, and one hundred and twenty minutes following intraperitoneal injection of 100 mg./Kg of salicylamide containing 30  $\mu$ c. of C-14 activity. Tissues taken for radioactivity analysis include the medulla, cerebellum, cerebral cortex, hypothalamic area, pituitary gland, cerebrospinal fluid, adrenal glands, and the plasma. The pituitary gland showed the greatest uptake of radioactivity of all tissues examined. Peak activity occurred at fifteen minutes for the pituitary, medulla, cerebrospinal fluid, and the adrenals. The hypothalamic area did not show a peak until sixty minutes. No well-defined peak was seen for the cerebellum and the cerebral cortex. The plasma did not exhibit its highest activity until thirty minutes, this time appearing to coincide with removal of the drug from the tissues.

The metabolism of C-14 carbonyl salicylamide was studied in normal and hypophysectomized rats. Following hydrolysis and ether extraction of the urine, paper chromatographic separation indicated the presence of salicylamide and gentisic acid amide. Free salicylamide was shown to be present in the unhydrolyzed urine while gentisic acid amide is apparently excreted entirely as a conjugate in this species. Gentisamide was

shown to occur in the urine to the extent of between 5 and 30 per cent.

The products obtained in the urine of the hypophysectomized animals were identical to those in the normal animals, indicating that the pituitary gland does not play a role in the hydroxylation of salicylamide.

In another group of animals the brain, pituitary gland, cerebrospinal fluid, adrenals, and plasma were examined for the presence of metabolic products. Paper chromatographic separation of ether extracts from these tissues showed the presence only of salicylamide. No gentisic acid amide was found in the tissues studied.

## REFERENCES

- (1) Hart, E. R., *J Pharmacol Exptl Therap*, **89**, 205 (1947)
- (2) Brodie, D. C., Way, E. L., and Smith, G., *THIS JOURNAL*, **41**, 48 (1952)
- (3) Gibson, R. D., Miya, T. S., and Edwards, L. D., *ibid*, **44**, 805 (1955)
- (4) Weikel, J., *ibid*, **47**, 477 (1958)
- (5) Seeburg, V. P., Hauser, D., and Whitney, B. J., *Pharmacol Exptl Therap*, **101**, 275 (1951)
- (6) Hernandez-Gutierrez, F., *Anales real acad farm*, **20**, 129 (1954)
- (7) Foye, W. O., Duvall, R. N., Lange, W. E., Talbot, M. H., and Prien, E. L., *J Pharmacol Exptl Therap*, **125**, 198 (1959)
- (8) Weygand, F., Becker, A., Feldmann, D., and Grossinsky, O. A., *Z physiol Chem*, **292**, 125 (1953)
- (9) Crabtree, R. E., Data, J. B., and Christian, J. E., *THIS JOURNAL*, **47**, 502 (1958)
- (10) Bousquet, W. F., and Christian, J. E., *ibid*, **47**, 542 (1958)
- (11) Bousquet, W. F., and Christian, J. E., *ibid*, **49**, 408 (1960)
- (12) Vaughan, M., Steinberg, D., and Logan, J., *Science*, **126**, 446 (1957)
- (13) Brodie, B. B., and Hogben, C. A. M., *J Pharm and Pharmacol*, **9**, 345 (1957)
- (14) Roth, L. J., Leiter, E., Hogness, J. R., and Langham, W. H., *J Biol Chem*, **176**, 249 (1948)
- (15) Bernstein, L., Veterans Administration Hospital, Denver, Colo., personal communication
- (16) Levine, R. M., and Clark, B. B., *J Pharmacol Exptl Therap*, **113**, 272 (1955)
- (17) Bray, H. G., Ryman, B. E., and Thorpe, W. V., *Biochem J*, **43**, 561 (1948)
- (18) Mitoma, C., Posner, H. S., Reitz, H. C., and Udenfriend, S., *Arch Biochem Biophys*, **61**, 431 (1956)
- (19) Mitoma, C., and Udenfriend, S., *J Pharmacol Exptl Therap*, **113**, 40 (1955)
- (20) Pallot, G., and Eberhardt, H., *Compt rend soc biol*, **180**, 1220 (1956)

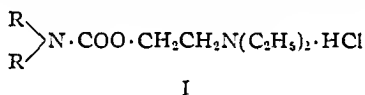
# Studies on Local Anesthetics XXV<sup>1</sup> (Physicochemical Properties of Drugs V<sup>2</sup>)\*

By ALEŠ SEKERA† and ČENĚK VRBA‡

The authors have studied the anesthetic activity of thirty diethylaminoethyl esters of substituted carbamic acids by surface and infiltration techniques and have attempted to correlate this activity with certain physicochemical properties, namely: surface tension activity, adsorbability, liposolubility, ability to coagulate colloids, and basicity of these compounds. The results obtained have been evaluated tabularly, graphically, and statistically. At the same time a summary has been given of the earlier results and they have been included in the discussion.

STUDIES OF THE RELATIONS between physicochemical properties and local anesthetic activity are fairly numerous (1). Their results have not only contributed to the elucidation of the mechanism of action of this group of drugs but also furnished some valuable hypotheses for the choice of the structures of new synthesized substances. It is essentially for this reason that we have given attention to these relations in our studies of the new local anesthetics of the series of basic carbamates.

On 30 substances of general formula I ( $R = H$ , alkyl, phenyl, tolyl, xylyl, alkoxyphenyl, naphthyl) (2) we have studied five physicochemical properties: surface tension (3), displacement



adsorbability (4), liposolubility (5), ability to coagulate colloids (6), and basicity (5). Easily accessible from the experimental point of view, they were chosen because of the importance which is attributed to them according to different theories of the mechanism of action of local anesthetics. They were compared with the relative activities of three kinds of anesthesia: surface, infiltration, and conduction (7), calculated as quotient of the durations of the complete anesthesia after application of the aqueous equimolar solutions of the substances studied and of those of the standards utilized (method A). The results obtained were graphically and statistically evaluated.

In our subsequent studies we have worked out a more precise method of testing surface and

infiltration anesthetic activity (8) based on determination and the comparison of the concentrations of the compounds studied, which have the same activity as the selected concentrations of the standards (method B). We have abandoned the study of conduction anesthesia because this type has similarities with infiltration anesthesia and because the method used is inaccurate. With the aid of this new method we have again studied the active substances of the series mentioned. The comparison of new values of the relative activities thus obtained with the values of the physicochemical measurements already determined constitute the subject of this communication. In view of a better comprehension of the results given we are presenting also the principles of the physicochemical methods utilized, with references to our earlier papers which contain detailed descriptions of them. This paper also summarizes some of the preceding results, which are presented in Tables I to III and are discussed and compared with our new results.

## EXPERIMENTAL

### Relative Surface Tension Measurements (3)

The relative surface tension activity was expressed by the relative values of the surface tension of 0.05 *M* aqueous solutions, buffered to pH 7.4. The measurements were made with Traube's stalagmometer, the laboratory temperature being 18 to 20°C. The relative values were calculated according to formula

$$\psi = \frac{a}{x} \cdot s$$

where  $a$  = number of drops of water (with stalagmometer used, 31.89),  $x$  = number of drops of the 0.05 *M* solution of the anesthetic, and  $s$  = density 20/4 (pycnometer method) of this solution.

**Relative Displacement Adsorbability (4).**—(One hundred milligrams ( $\pm 0.2$  mg.) of active charcoal (Ostakol, Spolek pro chemickou a hutní výrobu, Bohumín, Czechoslovakia) was shaken for an hour with a mixture of the aqueous solutions of 25 cc. of the anesthetic 0.04 *M* and 25 cc. of methylene blue

\* Received July 30, 1959, from the Department of Pharmaceutical Chemistry, Masaryk University, Brno, Czechoslovakia.

† Preliminary notes: *Naturwissenschaften*, 43, 303 (1956); *Biochem. Pharmacol.*, 2, 315 (1959).

‡ Present address: Service de Chimie, Laboratoire de Pharmacologie, 21 rue de l'Ecole de Médecine, Paris VI, France.

§ Department of Pharmacology, School of Veterinary Medicine, Brno, Czechoslovakia.

<sup>1</sup> Paper XXIV: *Arch. intern. pharmacodynamie*, in press.

<sup>2</sup> Paper IV: *Ann. pharm. franc.*, 16, 525 (1958).

TABLE I—PHYSICOCHEMICAL PROPERTIES OF INACTIVE DIETHYLAMINOETHYLESTERS OF SUBSTITUTE CARBAMIC ACIDS

No	R <sub>1</sub>	R <sub>2</sub>	Relative Surface Tension		Relative Displacement Adsorbability	Limit Molar Coagulating Concentration	Relative Liposolubility	pKa
			Non buffered	Buffered pH 7.0				
S 10	H	H	1 013	1 021	3 56	1 503	0 28	8 92
S 1	CH <sub>3</sub>	H	1 009	1 017	3 08	0 887	0 28	8 95
S 2	C <sub>2</sub> H <sub>5</sub>	H	1 005	1 007	3 42	0 887	0 24	8 94
S 21	CH <sub>3</sub>	CH <sub>3</sub>	1 008	1 003	2 54	1	0 36	8 92
S 19	Δ <sup>2</sup> -C <sub>5</sub> H <sub>9</sub>	H	1 007	0 998	2 66	0 4	0 24	(8 82)
S 3	C <sub>3</sub> H <sub>7</sub>	H	0 997	0 980	5 24	0 25	0 96	8 95
S 5	(CH <sub>3</sub> ) <sub>2</sub> CH	H	0 998	0 982	4 24	0 4	0 96	8 91
S 4	C <sub>4</sub> H <sub>9</sub>	H	0 979	0 939	7 26	0 094	2 24	8 93
S 7	CH <sub>2</sub> CH <sub>2</sub> CH(CH <sub>3</sub> )	H	0 982	0 956	7 26	0 122	0 24	8 95
S 8	(CH <sub>3</sub> ) <sub>2</sub> C	H	1 004	0 995	3 94	0 5	0 28	8 93
S 22	C <sub>2</sub> H <sub>5</sub>	C <sub>2</sub> H <sub>5</sub>	0 978	0 934	4 62	0 375	0 6	8 89
S 23	—CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> CH <sub>2</sub> —		0 987	0 956	5 16	0 225	5 6	8 87
S 24	—CH <sub>2</sub> CH <sub>2</sub> OCH <sub>2</sub> CH <sub>2</sub> —		1 006	0 998	3 26	1	0 4	8 74
S 9	C <sub>6</sub> H <sub>11</sub>	H	0 958	0 889	10 48	0 075	11 88	8 97

at 200 mg % After sedimentation the solution was filtered on a desorbed filter JENA 3G4, 30 cc was taken out and in the rest of the solution the methylene blue was determined colorimetrically. The relative values of the adsorbent power are expressed directly by the final concentrations of methylene blue found in the experiments.

**Relative Liposolubility (5).**—Twenty five cubic centimeters of the 0.01 M solution of the anesthetic, saturated with ether, was diluted with 50 cc of water saturated with ether. Then were added 10 cc of ether saturated with water and 25 cc of the phosphate buffer pH 7.4 saturated with ether. The mixture was shaken for ninety minutes. Five cubic centimeters of the ethereal layer was pipetted out, evaporated, and the residue was weighed after drying in a desiccator. The final values indicate the quantities of substances extracted in 10 cc of ether and are expressed in percentage of the base weighed.

**Ability to Coagulate Colloids (6).**—The ability to coagulate colloids was studied by determination of the limit molar concentrations of the anesthetics, coagulating the aqueous ethanol solution of Mastix, buffered at pH 7.0.

**Basicity (5).**—The basicity was studied by determination of the dissociation constants pKa. These were calculated from the pH of the aqueous solutions of the anesthetics of known concentrations of dissociated and nondissociated base.

## PHARMACOLOGY

### Determination of Local Anesthetic Activity.—

**Method A.**—The relative activities in surface anesthesia (rabbit's cornea, cocaine 0.05 M as standard), infiltration anesthesia (guinea pig, intradermal application, procaine 0.05 M as standard), and conduction anesthesia (frog's sciatic nerve, procaine 0.01 M as standard) were calculated as quotients of the durations of the complete anesthesia after application of the equimolar solutions of the substances studied and standards utilized.

**Method B.**—The relative activities in surface anesthesia (rabbit's cornea, cocaine 0.01 M as standard) and infiltration anesthesia (guinea pig, intradermal application, procaine 0.02 M as standard)

and) were calculated as quotients of the molar concentrations of the standards chosen and of the substances studied, having the same activity.

The detailed description as well as the evaluation of these two methods has been published elsewhere (7, 8). The final results presented in Tables I and II are taken in part from our earlier papers (2, 9) for purposes of comparison.

## STATISTICS<sup>3</sup>

The statistical evaluation of the relations studied was made by the calculation of Spearman's correlation coefficients (10).

The calculations were not made in the case of basicity because even by direct comparison no relation between the pKa and the anesthetic activities was found.

## DISCUSSION AND SUMMARY

The comparison of local anesthetic activity and five physicochemical properties (surface tension activity, adsorbability, liposolubility, ability to coagulate colloids, and basicity) has been made in the series of 30 basic carbamates. The results of these studies have been presented tabularly (Tables I and II), graphically (Figs 1 and 2), and statistically (Table III). In the tabular presentation the inactive substances were classified according to molecular weight and the active substances according to the place of substitution of the benzene nucleus of carbanilic acid. In the graphic presentation the active substances are arranged according to their activities.

The following relations between chemical structure, anesthetic activity, and physicochemical properties appear to exist:

1. The parallelism between the anesthetic activity and the physicochemical properties studied is apparent even in direct comparison. The physicochemical properties of the inactive aliphatic derivatives (Table I) differ clearly from the properties of the active aromatic derivatives, the latter manifesting a surface tension activity, an adsorbability, a liposolubility, and an ability to coagulate colloids distinctly higher.

<sup>3</sup> The authors wish to thank Dr. B. Chalupa for calculation of the correlation coefficients.



TABLE II.—RELATIONSHIPS BETWEEN THE ANESTHETIC ACTIVITIES AND THE PHYSICO-CHEMICAL PROPERTIES OF THE ACTIVE DIETHYLAMINOETHYLESTERS OF SUBSTITUTED CARBAMIC ACIDS

No	R <sub>1</sub>	R <sub>2</sub>	Relative Activity Calculated from the Duration of the				from the Equieffective Concentrations (Method B)				Relative Displacement Adsorbability	Limit Molar Coagulating Concentration	Relative Liposolubility	pK <sub>a</sub>
			Surface Anesthesia	Infiltration Anesthesia	Conduction Anesthesia	Surface Anesthesia	Infiltration Anesthesia	Non-buffered	Surface Tension pH 7.0	Buffered				
S 11	C <sub>6</sub> H <sub>5</sub>	II	0.32	1.4	2.8	0.1	1.2	0.993	0.943	→	28.2	0.0112	10.8	8.81
S 12	<i>m</i> -C <sub>6</sub> H <sub>4</sub> CH <sub>3</sub>	II	0.52	3.3	2.5	0.67	3.4	0.921	0.802	→	65.8	0.00916	21.8	8.76
S 25	C <sub>6</sub> H <sub>5</sub>	II	0.8	4	1.9	1.4	3.6	0.869	0.661	→	45.4	0.00892	30.2	8.81
S 31	2-ClH <sub>3</sub> C <sub>6</sub> H <sub>4</sub>	II	0.46	1.1	2.4	0.54	1	0.989	0.929	→	24.3	0.0125	9.8	8.81
S 35	2-ClH <sub>3</sub> O·C <sub>6</sub> H <sub>4</sub>	II	0.5	1.5	2.6	0.33	1.7	0.988	0.931	→	30	0.033	11.4	8.84
S 38	2-C <sub>6</sub> H <sub>5</sub> O·C <sub>6</sub> H <sub>4</sub>	II	0.72	3	3.8	0.31	2.1	0.967	0.856	→	38.9	0.0175	20.8	8.82
S 41	2-C <sub>6</sub> H <sub>5</sub> O·C <sub>6</sub> H <sub>4</sub>	II	3	3	2.6	21	42	0.926	0.440	→	68.8	0.00063	38.9	8.68
S 36	3-ClH <sub>3</sub> O·C <sub>6</sub> H <sub>4</sub>	II	0.13	1.5	0.8	0.13	0.64	0.976	0.909	→	43.2	0.0175	11.1	8.77
S 32	3-ClH <sub>3</sub> C <sub>6</sub> H <sub>4</sub>	II	0.43	1.5	1.6	0.26	1.3	0.952	0.832	→	37.1	0.0125	18.2	8.84
S 39	3-C <sub>6</sub> H <sub>5</sub> O·C <sub>6</sub> H <sub>4</sub>	II	0.82	2.4	3.2	0.46	1.7	0.913	0.746	→	50	0.010	20.5	8.76
S 15	3-C <sub>6</sub> H <sub>5</sub> O·C <sub>6</sub> H <sub>4</sub>	II	4	2.8	1.1	50	70	0.881	0.483	→	83.2	0.00218	39.4	8.68
S 37	4-ClH <sub>3</sub> O·C <sub>6</sub> H <sub>4</sub>	II	0	0.8	0.8	0	0.86	0.983	0.941	→	35	0.055	7.8	8.81
S 10	4-C <sub>6</sub> H <sub>5</sub> O·C <sub>6</sub> H <sub>4</sub>	II	0.13	1	0	0.02	0.87	0.938	0.802	→	46.4	0.0195	13.1	8.80
S 31	2,4-(ClH <sub>3</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>3</sub>	II	0.27	1.2	1.1	0.21	1.34	0.939	0.799	→	21.9	0.0175	14.2	8.82
S 33	4-ClH <sub>3</sub> C <sub>6</sub> H <sub>4</sub>	II	0.31	1.2	1.7	0.03	1.24	0.947	0.828	→	25.2	0.0156	16.6	8.85
S 16	1-C <sub>6</sub> H <sub>5</sub> O·C <sub>6</sub> H <sub>4</sub>	II	1.3	1.3	1.3	1.4	3	0.928	0.482	→	80	0.00188	37.9	8.68

<sup>a</sup> The substance being prepared as the base, the pK<sub>a</sub> was not determined. In the case of the isomeric  $\beta$ -naphthyl derivative pK<sub>a</sub> = 8.67.

In the case of the aromatic derivatives the relationships appear still more distinctly in the groups formed according to the position of substituents in the benzene nucleus of carbamyl acid (Table II); in the homologous series of the alkoxy derivatives they approach the quantitative relations.

It seems interesting to note that with the formation of the groups according to the substituents (position isomers) the relations studied are partially decreased. This phenomenon might be associated with the supposition that the position isomery influences relatively little the processes of adsorption, the liposolubility, and the ability to coagulate colloids, and intervenes more noticeably in the other physicochemical properties, e. g., the stability of the carbamic group of carbamyl acid (metabolism), which depends upon the distribution of the electrostatic charge of the molecule varying with the position of the substituent in the benzene nucleus.

2. It is striking to note the parallelism of the determined values of surface tension activity, adsorbability, liposolubility, and the ability to coagulate colloids when compared among themselves. The relation between surface tension activity and adsorbability is easily explained, for it is a question of the same physicochemical process differing only in the nature of the phases. Also understood is the affinity of these adsorbabilities at the contact of two phases with the liposolubility, affinity already pointed out by Traube in the case of narcotics (11).

The parallelism of the limit concentrations coagulating the Mastiv colloidal solution seems to indicate that in the series studied this physicochemical property is close to the processes of adsorption and less dependent on the electrostatic charge of the molecule (pK<sub>a</sub>). This fact concurs also with the relations found in the group of the narcotics (12).

3. A relationship between anesthetic activity and basicity was not found. This probably results from the fact that the pK<sub>a</sub> of the substances studied differ only very little from each other and that they have rather high values; at pH 7.4 of the biological substrate all the substances are dissociated within 96–98%. The influence which they exercise on the other physicochemical properties is slight because of these very feeble differences.

4. The results obtained by the graphical representation correspond to those found by use of the tabular classification; the relationships between the anesthetic activity and the

TABLE III.—STATISTICAL EVALUATION OF THE RELATIONS STUDIED  
(SPEARMAN'S CORRELATION COEFFICIENT)

Physicochemical Property	Relative Activity Calculated—				
	Surface Anesthesia	from the Duration of the Infiltration Anesthesia (Method A)	Conduction Anesthesia	Surface Anesthesia	from the Equieffective Concentrations (Method B)
Surface tension activity (nonbuffered solutions)	0.745 ± 0.057	0.776 ± 0.052	0.601 ± 0.083	0.759 ± 0.054	0.798 ± 0.047
Surface tension activity (buffered solutions)	0.852 ± 0.035	0.817 ± 0.043	0.716 ± 0.063	0.853 ± 0.035	0.869 ± 0.031
Displacement adsorbability	0.880 ± 0.029	0.879 ± 0.029	0.742 ± 0.058	0.865 ± 0.033	0.892 ± 0.026
Distribution coefficient	0.882 ± 0.029	0.878 ± 0.030	0.767 ± 0.053	0.881 ± 0.029	0.914 ± 0.021
Ability to coagulate colloids	0.905 ± 0.023	0.886 ± 0.028	0.817 ± 0.043	0.912 ± 0.022	0.913 ± 0.021

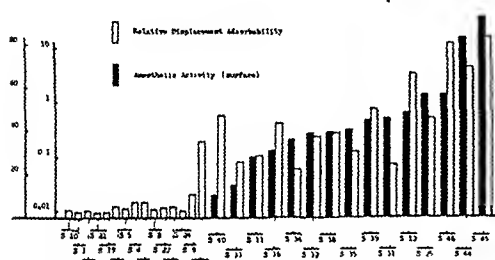


Fig. 1.—Relationship between the relative displacement adsorbability and the surface anesthetic activity (method B)

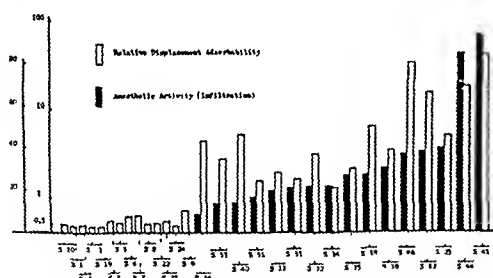


Fig. 2.—Relationship between the relative displacement adsorbability and the infiltration anesthetic activity (method B).

physicochemical properties are distinct without being quantitative. As an example we present the graph of the relations between the surface anesthetic activity (method B) and the displacement adsorbability (Figs 1 and 2).

5. Statistical evaluation showed a clearly positive relation between the anesthetic activity and the physicochemical properties, which follows from the high values of the correlation coefficients (Table II). The distinctly lower values in the case of conduction anesthesia (method A) are probably due to the fact that the method utilized furnished results difficult to reproduce and rather dispersed.

From the statistical results it seems also that the ability to coagulate colloids is an important property in the mechanism of local anesthetic action. The increase of the correlation coefficients in the

is striking; surface tension activity (nonbuffered solutions) < surface tension activity (buffered solutions) < displacement adsorbability, which seems to concur with the progressively closer relationship between these model experiments and the process of adsorption taking place in the organism.

In tabular and even in graphical comparison the conclusions obtained with the new method of testing (method B) differ very little from those deduced from the pharmacological results obtained with the earlier method of testing (method A). Only statistical evaluation showed the distinct difference. The correlation coefficients of the physicochemical properties with the relative activities obtained by the new method are, in general, higher. This results, probably, from the greater precision of the pharmacological method (see, on the other hand, the low values of correlation coefficients in the case of the inexact method of conduction anesthesia, already mentioned).

The theoretical results obtained have also found a practical use in the choice of the structures of new synthesized substances. They made evident the advantage of alkoxylation which increases the liposolubility and the other physicochemical properties studied and similarly the local anesthetic activity. This influence grows with the prolongation of the carbon chain of alkoxy. The knowledge of this fact, seen already in the studies of the methoxyl and ethoxyl derivatives S 35 to S 40, led us to the synthesis of the butoxyl derivatives S 44 to S 46. Only later these substances were included in the studies of physicochemical properties. In the framework of our following research the same reason led us to the profound study of the influence of alkoxylation, aralkoxylation, and aroxylation in the series of basic carbamates and anilides. In the course of these studies we have been able to prepare several relatively active substances with a fairly low order of toxicity which were, in part, the subject of our previous publications (13–15).

## REFERENCES

- (1) Laubener, W., "Hefter's Handbuch der experimentellen Pharmakologie," Ergänzungswerk Bd. VIII, Springer, Berlin, 1939, p. 16; Löfgren, N., "Xylocaine, a New Synthetic Drug," Ivar Hoegsströms Boktryckeri A. B., Stock-

holm, 1948, Soebring, K., *Pharmazie*, 4, 359(1949), Büchi, J., *Bull. fédération intern. pharm.*, 21, 179(1947), 23, 3(1949), *Arzneimittel Forsch.*, 2, 1, 65, 114(1952)

(2) Sekera, A., Hrubý, J., Vrba, Č., and Lebduška, J., *Českoslov farm.*, 1, 12(1952), Sekera, A., Jakubec, I., Král, J., and Vrba, Č., *Chem listy*, 46, 762(1952), Sekera, A., Borovanský, A., and Vrba, Č., *ibid.*, 47, 591(1953), Sekera, A., Borovanský, A., Jakubec, I., Palat, K., and Vrba, Č., *Českoslov farm.*, 5, 388(1956)

(3) Sekera, A., Jakubec, I., and Vrba, Č., *Českoslov farm.*, 5, 462(1956)

(4) Sekera, A., Jakubec, I., and Vrba, Č., *ibid.*, 6, 31(1957)

(5) Sekera, A., Borovanský, A., and Vrba, Č., *Ann pharm franç.*, 16, 525(1958)

(6) Sekera, A., Kral, J., and Vrba, Č., *Českoslov farm.*, 6, 197(1957)

(7) Vrba, Č., Lebduška, J., and Sekera, A., *ibid.*, 1, (1952).

(8) Vrba, Č., and Sekera, A., *Arch intern pharmacol.*, 118, 155(1959), Roth, Z., *ibid.*, 118, 289(1959)

(9) Palát, K., Sekera, A., and Vrba, Č., *Chem listy*, 563(1957), Sekera, A., and Vrba, Č., *Arch pharm.*, 291, (1958).

(10) Kendall, M. G., "The Advanced Theory of Statistics," Griffin, London, 1943

(11) Traube, J., "Die Narkose," 2nd ed., Springer, Ber 1926, pp 325, 342

(12) Winterstein, H., *loc cit*, p 334

(13) Dofek, R., Sekera, A., and Vrba, Č., *Tris Journ.*, 48, 398(1959)

(14) Sova, J., Sekera, A., and Vrba, Č., *Chem listy*, 2339(1957)

(15) Borovanský, A., Sekera, A., and Vrba, Č., *J. JOURNAL*, 49, 57(1960)

## The Design of a Continuous Recording *In Vivo* Method of Measuring Sensible Perspiration Over a Limited Area<sup>+</sup>

By WILLIAM J. O'MALLEY† and JOHN E. CHRISTIAN

The objective of this study was to develop a continuous recording, *in vivo* method of measuring sensible perspiration over a limited area. The study required the design and construction of a suitable electrical circuit and electrolytic cell containing a methanol-acetone-oxalic acid mixture which was sensitive to small amounts of water. The immediate desired application was for the evaluation of antiperspirant preparations. The perspiration was collected by passing dry nitrogen gas through a skin cell which enclosed a small area of skin and delivered the moisture-laden gas to the electrolytic solution. Two such systems were combined through suitable electrical connections to compare, simultaneously and continuously, two adjacent areas of skin.

THE MAIN OBJECTIVE of this study was to develop a continuous recording, *in vivo* method of measuring sensible perspiration over a limited area of the body. The immediate desired application was for the evaluation of antiperspirant preparations, however, the method should also be applicable to the evaluation of any drug which affects the rate of perspiration flow.

The electrical conductivity of mixed organic solvents has been used to measure water vapor (1-4). Such electrical conductivity measurements require the use of a d c Wheatstone bridge as the means of measuring the change in the resistance of the electrolytic cell as water is added (5-7). Bright platinum electrodes (8) are required in the presence of organic solvents such as methanol, acetone, and 1,4-dioxane to prevent redox reactions at the electrodes. Weak

electrolytes have the advantage, over strong electrolytes, of being more soluble in the organic solvents (9). In addition, weak electrolytes are not dissociated in the organic solvents and are only slightly dissociated in water, therefore, concentration in these systems is not a critical factor (10).

### EXPERIMENTAL

This study required the design and construction of suitable circuits and electrolytic cells containing a methanol-acetone-oxalic acid mixture which is sensitive to small amounts of water. The perspiration was collected by passing dry nitrogen gas through a skin cell which enclosed two adjacent areas of skin and delivered the moisture-laden gas to the electrolytic solutions.

The initial experimental phase of this study involved the design and development of the continuous recording instrumentation which permitted the measurement of small quantities of water vapor (sensible perspiration) in gases. To permit the study of two adjacent areas, two separate systems were combined; one gaseous, to carry the moisture from the skin to the electrolytic cell, and the other electrical, to measure the moisture added to the

\* Received August 21, 1959, from Purdue University, School of Pharmacy, Lafayette, Ind.

† Present address: Medical College of Virginia, School of Pharmacy, Richmond 19.

Presented to the Scientific Section, A. P. A., Cincinnati meeting, August 1959.

electrolytic cell. The electrolytic solution was sensitive to very small (0.010 ml) quantities of water. The water thus collected was measured by noting the decrease in electrical resistance using a d c Wheatstone bridge. Since it was desirable to compare two adjacent areas continuously and simultaneously, it was necessary to have two electrolytic cells, two nitrogen supply lines, and two bridge circuits. The two bridges were connected to a single point recorder for continuous, simultaneous recording. Figure 1 gives a schematic diagram of the dual system used in these studies.

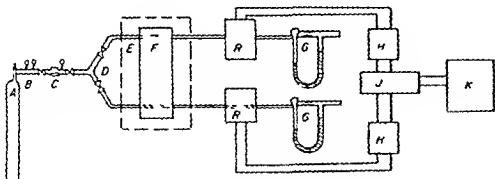


Fig 1—A schematic of the dual system. A, dry nitrogen gas, B, double stage regulator, C, low pressure regulator, D, Y-connection, E, constant temperature booth, F, skin cell, G, differential flow-meters, H, d c Wheatstone bridges, J, timing mechanism and changeover circuit, K, single point recorder, R, electrolytic cells

**The Bridge.**—A d c Wheatstone bridge was found satisfactory for measuring the decrease in resistance resulting from the addition of water to an electrolytic cell. The voltage was supplied by four, 1½ volt dry cell batteries connected in series. The batteries and the detector were interchanged (7) to enable a more rapid standardization of the bridge. A variable resistor, in series with the batteries, was used to regulate the current flow and prevent over loading of the circuit as well as controlling the sensitivity of the bridge to water. The known resistances were a L N AC-DC decade resistance box (No 4754)<sup>1</sup> and a L-N slidewire box (No 4261).<sup>1</sup> A rubber stopper was found suitable to hold the electrodes and the gas inlet and outlet tubes.

The gas tubes were made from 4 mm i d Pyrex glass tubing and the electrodes were constructed by sealing 24 gauge platinum wire into 4 mm i d Pyrex glass tubing so that 5 cm of the wire extended out from the point of sealing. A 3 cm portion extended inside the tube and was joined to the bridge through a mercury pool type contact. The exposed 5 cm length was wound in a tight, flat coil of about 1 cm in diameter. The optimum distance between the electrodes was found to be 5 mm. The gas inlet tube was placed equidistant from both electrodes with the orifice turned away from the electrodes. Since in this study it was only necessary to determine the change in the resistance of the solvent-electrolyte system, the cell constant was not determined.

A number of solvent-electrolyte systems, previously reported, were studied using this bridge circuit and a L-N millivolt recorder (Speedomax type G model S, 60000 Series)<sup>1</sup> as the indicating device (1-4, 11-16). The systems were prepared in varying proportions and carefully tested for electrical response to 0.010 ml aliquots of water added

directly to the cell which was maintained at  $21.0 \pm 0.1^\circ$  in a water bath. Each electrolyte was carefully dried prior to testing.

**The Gaseous System.**—High purity dry nitrogen gas<sup>2</sup> carried water vapor (perspiration) from a vapor cell or a skin cell to the electrolytic cell and, at the same time, stirred the mixture. All of the connections in the gaseous system were of either Pyrex glass or Tygon tubing.<sup>3</sup> Rubber was avoided since it has a tendency to absorb moisture. The pressure was regulated by a double stage tank gauge and a low pressure "pancake" type diaphragm valve.<sup>4</sup>

In the initial work only one bridge was standardized at a time, therefore, during the standardization procedure only one arm of the gaseous system was used. In the complete dual system the gas pressure was adjusted in either arm of the gaseous system by the stopcocks in the arms of the Y-connection. Minor evaporation of the electrolytic solvents was noted but this did not affect the results.

A vapor and a skin cell were made to enclose a given volume of water or area of skin from methyl methacrylate plastic.<sup>5</sup> The vapor cell contained a single groove 7 cm long, 0.5 cm wide, and 0.5 cm deep, with suitable inlet and outlet orifices. The skin cell was of similar construction but consisted of two grooves which were intended to enclose two adjacent areas 1 cm apart. A second plate was used to seal the skin cell when it was not enclosing an area of skin. In the vapor cell the second plate had a similar groove which represented the area of the skin covered. The cells were made airtight by applying a thin layer of petrolatum to the contact surface of the bottom plate or by applying mild pressure to the cell resting on the surface of the body.

**The Dual Electrical Circuit.**—Figure 2 shows the dual electrical system used to study a treated and an untreated area of the skin simultaneously. There are two d c Wheatstone bridges connected through a double pole, double throw (DPDT) relay<sup>6</sup> to the single point recorder. The source of power in each bridge was four 1½ volt dry cell batteries. The bridges differed only in that a second slidewire box was not available, so two decade resistance boxes were connected in parallel and served the same purpose in the second bridge. The bridges were wired with No 18 gauge, single strand wire (No 8945)<sup>7</sup> and the leads from the DPDT relay were shielded wire (No 8410)<sup>7</sup> because of the distance between the relay and the recorder. A DPDT relay was used as an inexpensive means of converting the single point recorder to a multipoint unit. The activating circuit of the DPDT relay consisted of a 45 volt dry cell battery connected in series with a momentary contact switch, *Km*. The momentary contact switch was activated by a cam drive which was connected to a small electrical motor through a pulley drive mechanism. The cam had a speed of 2 r p m and was cut in such a manner as to activate the DPDT relay for two thirds of its cycle. This causes one bridge to record for one-third of the cycle of the cam and the other bridge for two thirds of the

<sup>1</sup> Linde Air Products Co., Indianapolis, Ind.

<sup>2</sup> The United States Stoneware Co., Akron, Ohio.

<sup>3</sup> Regulator No. 70, The Matheson Co., Joliet, Ill.

<sup>4</sup> Rohm and Haas Co., Philadelphia, Pa.

<sup>5</sup> Type LM11, Potter and Brumfield Co., Prince

<sup>6</sup> Belden Wire Co., Chicago, Ill.

<sup>7</sup> Leeds Northrup Co., Philadelphia, Pa.

TABLE I—A COMPARISON OF THE VOLUME OF WATER COLLECTED FROM THE VAPOR CELL TO THAT INDICATED BY A CHANGE IN THE RESISTANCE OF THE ELECTROLYTIC CELL

Electrolytic Cell $R_1$					Electrolytic Cell $R'_1$				
Time <sup>a</sup>	mV <sup>b</sup>	OV <sup>c</sup>	Microliters	CV <sup>d</sup>	Time <sup>a</sup>	mV <sup>b</sup>	OV <sup>c</sup>	Microliters	CV <sup>d</sup>
60	0 40	30 0	33 0	33 0	30	0 90	30 0	39 0	39 0
60	0 39	30 0	32 0	32 0	30	0 44	20 0	19 0	19 0
60	0 64	40 0	43 0	43 0	32	0 93	28 0	41 0	41 0
45	0 39	30 0	32 0	32 0	30	0 77	25 0	34 0	34 0
30	0 32	30 0	28 0	28 0	30	0 59	28 0	26 0	26 0
30	0 30	30 0	25 0	25 0	30	0 91	28 0	40 0	40 0
50	0 52	30 0	43 0	43 0	30	0 62	29 0	27 0	27 0
60	0 52	30 0	42 0	42 0	45	0 78	40 0	34 0	34 0
45	0 31	35 0	26 0	26 0	30	0 83	30 0	36 0	36 0
30	0 54	30 0	45 0	45 0	30	0 75	28 0	33 0	33 0
30	0 47	25 0	39 0	39 0	30	0 62	22 0	27 0	27 0
					30	0 96	28 0	42 0	42 0
					34	1 05	30 0	46 0	46 0
Σ		340		388			366		444
μ <sup>e</sup>		30 9		35 3			28 2		34 2
σ <sup>f</sup>		3 75		7 30			4 50		7 70
μ <sub>D</sub> <sup>g</sup>			33 1					31 2	
σ <sub>D</sub> <sup>h</sup>			2 51					2 47	

- <sup>a</sup> Time in minutes  
<sup>b</sup> Millivolts response per unit time  
<sup>c</sup> Observed value the volume in  $\mu\text{L}$  as indicated by a change in resistance of the recorder  
<sup>d</sup> Calculated value the volume in  $\mu\text{L}$  as determined by the loss in weight of the vapor cell, converted to volume  
<sup>e</sup> Mean  $n\mu = \sum_{j=1}^n X_j$   
<sup>f</sup> Standard deviation  $(n-1)\sigma^2 = \sum_{j=1}^n (X_j - \mu)^2$   
<sup>g</sup> Difference of two means  $n\mu_D = \sum_{j=1}^n \mu_j$   
<sup>h</sup> Standard deviation of the difference of two means  $\sigma_D^2 = \sigma^2 + \sigma'^2$

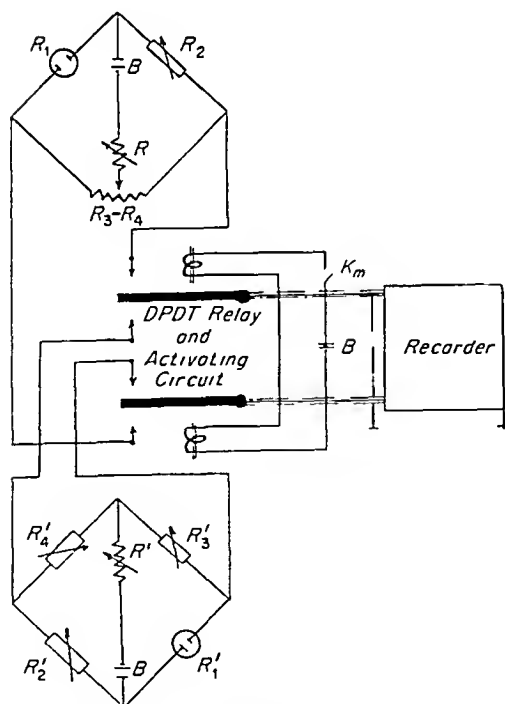


Fig 2—The dual Wheatstone bridge and change-over circuit

cycle of the cam. This also supplied a means of differentiating between the two bridges and the two areas of skin, on one recording.

## RESULTS

**Electrolytes and Solvents.**—Two electrolytes were studied with different solvents, NaCl A R, which was dried at 140° for twenty-four hours and oxalic acid A R, which was dried at 103° for twenty-four hours. The different solvent systems tested with a saturated solution of NaCl were methanol, methanol benzene, methanol dioxane, and methanol acetone. The solvent systems used with oxalic acid were methanol, dioxane, methanol dioxane, and methanol-acetone. In this case both the solvent and the electrolyte were varied extensively to obtain the most reproducible results.

The most effective solvent electrolyte system was 25.0 ml methanol (tech.), 5.0 ml acetone N F, and 1.0 Gm oxalic acid. It was also found that the oxalic acid in the methanol-acetone mixture was not a critical factor, therefore, the amount of oxalic acid in solution could be varied by  $\pm 0.010$  Gm without appreciably affecting the results. Another advantage of oxalic acid was its complete solubility in the organic solvents in the concentrations used.

**Standardization of the Electrolytic Cells.**—The cell bridge combination had its own special properties which may not be identical with a similar combination, therefore, the two cells,  $R_1$  and  $R'_1$ , were studied separately and a calibration curve was determined for each cell by adding 0.010 ml (10  $\lambda$ ) aliquots of water from a 10  $\lambda$  pipet directly to the electrolytic cell. Figure 3 shows the response curves for each cell. Seven determinations were used to calculate each point on the response curves for  $R_1$  and  $R'_1$ . The average responses for  $R_1$  and  $R'_1$  were 0.121 mV / 0.010 ml and 0.225 mV / ml, respectively over the range of 0 to 0.150 ml of  $\text{H}_2\text{O}$ . The re-

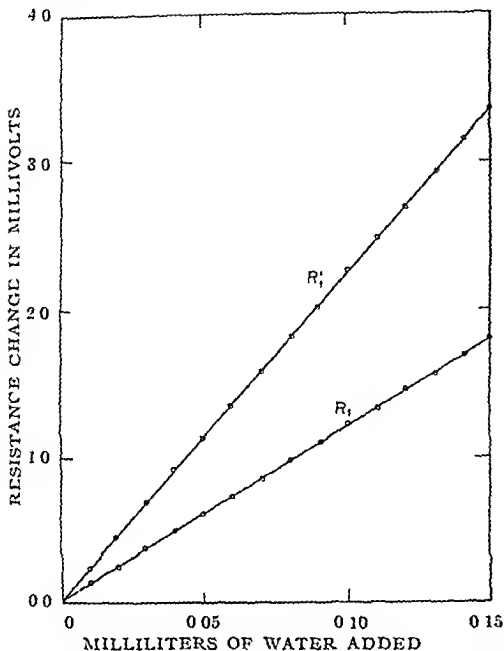


Fig 3—The relationship between the resistance change and 0.010 ml per aliquot of water

for the variation in these values was that the two bridges must standardize at the same time and maintain parallel baselines on the recorder for the two tests to be conducted simultaneously

**Comparison of the Vapor Cell with an Electrolytic Cell.**—To prove that the procedure developed in this study was an analytical method of measuring moisture in gases, it was necessary to develop a cell in which known weights of water could be placed and the loss of weight (water) determined as a stream of gas passed over the water. Table I is a comparison of the loss of weight of the water sample with the amount of water absorbed by the electrolytic solution, as indicated by the change in resistance of the bridge. The temperature surrounding the vapor cell was  $40.0 \pm 1.0^\circ$ . The weight of water was converted to volume.

## SUMMARY AND CONCLUSIONS

1 A conductance method has been demonstrated for the measurement of water vapor in gases and is intended to be applied to the determination of the rate of perspiration flow from treated and untreated areas of the body

2. Several combinations of solvent-electrolyte systems were screened for use in this investigation and methanol (tech.), 25.0 ml.; acetone N. F. 5.0 ml.; and oxalic acid A. R., 1.0 Gm. was selected as providing the most reproducible results

3 Using the principles of the method developed, a dual system involving a dual bridge circuit was constructed which, in combination with a specifically designed cell, allowed two areas or samples to be studied continuously and simultaneously.

## REFERENCES

- (1) Bocke, J., *Philips Tech Rev*, **9**, 13(1947)
- (2) Venkatanarasimhachar, N., *Proc Indian Acad Sci*, **16A**, 332(1942)
- (3) Hancock, C. K., and Hudgens, C. M., *Anal Chem*, **26**, 1738(1954)
- (4) Burton, M. B., Jr., M.S. thesis, Texas A and M, Lubbock, Texas, January 1953
- (5) Dawes, C. L., "Industrial Electricity," Vol. I, 3rd ed, McGraw-Hill Book Co., New York, N. Y., 1956, p. 126
- (6) Hausemann, E., and Slack, E. P., "Physics," D. Van Nostrand Co., New York, N. Y., 1948, p. 388
- (7) Creighton, H. J., "Principles and Applications of Electrochemistry," Vol. I, 4th ed, John Wiley & Sons, New York, N. Y., 1943, p. 67
- (8) *Ibid*, p. 236
- (9) Dole, M., "Electrochemistry," McGraw-Hill Book Co., New York, N. Y., 1935, p. 42
- (10) Daniels, F., *et al*, "Experimental Physical Chemistry," 4th ed, McGraw-Hill Book Co., New York, N. Y., 1949, p. 161
- (11) Kraus, C. A., and Fuoss, R. M., *J Am Chem Soc*, **55**, 21(1933)
- (12) Lichtenwalter, H. O., and Cody, H. P., *ibid*, **35**, 1434(1913)
- (13) Taylor, H. S., "Treatise on Physical Chemistry," D. Van Nostrand Co., New York, N. Y., 1931, p. 723.
- (14) Meites, L., "Polarographic Techniques," Interscience Publishers, New York, N. Y., 1955, p. 144
- (15) Komarov's, V. A., Russian pat 51,904 (Oct 31, 1937), *Anal Chem*, **26**, 1738(1954)
- (16) Bunge, M., *J Chem Soc*, **30**, 286(1876), **31**, 455(1877)

# An Evaluation of the Ability of Antiperspirant Compounds to Reduce Perspiration Flow\*

By WILLIAM J. O'MALLEY† and JOHN E. CHRISTIAN

The objective of this study was to apply a previously developed electro-conductivity method of measuring moisture in gases and vapors to the determination of the ability of an antiperspirant compound to reduce perspiration flow. A skin cell was developed which enclosed two adjacent areas of skin. The optimum site of application of the skin cell was the forearm and the optimum environmental temperature of the subject was 35°. Three types of commercial bases were studied to determine their effect on the perspiration flow. Then six different commercial products and one compound not commercially available were compared to 24 per cent  $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$  solution to show their effectiveness at reducing perspiration flow.

**A**N ELECTRO-CONDUCTIVITY method previously developed for measuring moisture in gases and vapors (1) was applied to the measurement of the effectiveness of several antiperspirant compounds to reduce sensible perspiration flow from a restricted area of the body.

Most of the previous methods used did not require a precise knowledge of the temperatures producing insensible perspiration, sensible perspiration, and profuse local perspiration, since they were *in vitro* methods. These methods can be divided between those which precipitate proteins (2, 3, 4) and those which adsorb water on an insoluble material (5-8). There were only two methods which simulated air currents, Palmes (9) used infrared analysis, and Hill and Hargood-Ash (10) measured the thermal conductivity of water vapor. In both of these cases a gas was passed over an enclosed area of the skin.

Using the electro-conductivity method it was necessary to determine the optimum temperature at which sensible perspiration was eliminated through the subject's sweat glands and ducts (11) but at which profuse local perspiring does not occur, and an optimum site of application of a skin cell.

A temperature of 35° was selected since it was sufficiently below the temperature necessary to stimulate profuse local sweating in those areas containing apocrine glands (12). The rate of profuse local sweating was such that it exceeded the ability of the gaseous system to evaporate the perspired water. The forearm was selected as the site of application of the skin cell since it was easily accessible in both men and women.

After having determined the optimum temperature and site of application of the skin cell, it was necessary to show that the amount of water

collected from two adjacent areas at the same time was equal. Then three types of bases were studied to prove that the commercial bases did not affect the antiperspirant activity of the compounds, and finally, six different commercial products and one compound not commercially available were compared with 24 per cent w/w  $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$  solution.

## EXPERIMENTAL

**Apparatus.**—The preparation of the electrolytic cells and the apparatus was previously described (1).

**Preparation of the Chemical Standard.**—It was advisable to select a standard of comparison which would be easily obtainable, one which had been used before (5), and one which could be used at a later date with confidence that there were no hidden elements to affect the results. For these reasons,  $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$  was used in the form of a 24% w/w solution. The aluminum sulfate (24.0 Gm.) was dissolved in 75.8 Gm. of distilled water and 0.2 Gm. of Tween 20 to make 100 Gm. of a 24% w/w solution.

**Preparation of Treated Areas of the Forearm.**—The forearm was divided into a front and a back area. Each of these areas was then divided into two parts longitudinally, and the separate areas were marked off with a solution of silver nitrate. To the left side, in each case, was applied 0.07 ml. of aluminum sulfate solution. The other side went untreated or was treated with 90 mg. of cream or stick, or 0.07 ml. of lotion. The bases and the finished products (creams, sticks, and lotions) were representative commercial products. The solution of aluminum sulfate, the commercial bases and products, and the research compound were applied once each day for the duration of the test. The areas were washed each morning with a mild facial soap.

## RESULTS

**The Determination of Sites of Application.**—There are three types of stimuli which cause perspiring in man; thermal, mental, and a combination of thermal and mental. These three types of stimuli produce perspiration at different areas on the body, and since the factors which affect mental perspiration are so varied from one individual to another, the areas of mental perspiring should be avoided when

\* Received September 25, 1959, from Purdue University, School of Pharmacy, Lafayette, Ind.

† Present address: Medical College of Virginia, School of Pharmacy, Richmond.

testing antiperspirants, even though some of these areas are common points of antiperspirant application (axillae). A broad surface which was easily accessible to both men and women was the forearm.

**The Determination of the External Temperature.** It was found the results were very erratic if only a portion of the body was submitted to a higher temperature, therefore, a special constant temperature booth was constructed for use during the experiment. Experiments were run at 25, 35, 40, and  $45^{\circ} \pm 1^{\circ}$ . The data obtained at  $35^{\circ}$  are shown in Table I. The ratio is of the left area to the right area under the skin cell, and the test time was fifteen minutes.

TABLE 1.—A COMPARISON OF PERSPIRATION FLOW FROM LIMITED ADJACENT AREAS OF SKIN AT  $35^{\circ}$

Left <sup>a</sup>	Right <sup>a</sup>	Ratio <sup>b</sup>
8 0	11 0	0 73
12 0	10 0	1 24
17 0	16 0	1 09
14 0	15 0	0 93
11 0	11 0	1 00
8 0	8 0	1 00
16 0	16 0	1 00
14 0	16 0	0 88
15 0	13 0	1 15
17 0	15 0	1 13
6 0	7 0	0 91
12 0	13 0	0 94
11 0	12 0	0 93
$\Sigma$ 161 0	163 0	12 930
$\mu^c$ 12 38	12 53	0 994
$\sigma^d$ 3 73	2 92	0 2135

<sup>a</sup> The number of  $\mu$ L of water collected in fifteen minutes

<sup>b</sup> Ratio of left to right

<sup>c</sup> Mean  $\mu = \sum_{j=1}^n X_j$ , where  $n = 13$

<sup>d</sup> Standard deviation:  $(n-1)\sigma^2 = \sum_{j=1}^n (X_j - \mu)^2$

At  $35^{\circ}$  the atmosphere was sufficiently warm to induce a uniform flow of sensible perspiration from adjacent areas of the body (ratio = 0.994). Above this temperature the severe heat produced such unwanted effects as emotional unrest and excessive perspiration flow which the gas was not able to carry away, while below this temperature the amount of perspiration released was not enough to indicate reproducible results from adjacent areas.

**The Comparison of Normal Skin with 24% w/w  $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$  Solution.**—Table II shows the microliters of water collected from two adjacent areas of the forearm and indicates the ratio of water collected from normal and aluminum sulfate-treated skin. The average value of the ratio of normal skin to aluminum sulfate-treated skin indicates that 1.94 times as much water was lost from normal skin as from aluminum sulfate-treated skin.

**The Evaluation of Antiperspirant Bases.**—Three types of commercial bases and normal skin were compared with 24% w/w  $\text{Al}_2(\text{SO}_4)_3 \cdot 18 \text{H}_2\text{O}$  solution. Table III indicates that approximately the same amount of water was collected from normal skin as was obtained from normal skin treated with a stick base, a cream base, and a lotion base. The results also indicate that the ratio between normal skin and aluminum sulfate-treated skin was similar to the ratio between the areas treated with the three bases

TABLE II.—A COMPARISON OF THE AMOUNTS OF WATER COLLECTED FROM A LIMITED AREA OF NORMAL AND 24% w/w ALUMINUM SULFATE-TREATED SKIN

Normal	Aluminum Sulfate	Ratio
11 5	6 7	1 72
8 3	5 9	1 41
15 6	10 1	1 54
6 6	4 2	1 57
11 5	4 2	2 74
9 3	5 0	1 86
12 5	5 0	2 25
11 1	5 9	1 88
16 0	7 6	2 11
9 7	4 2	2 31
$\Sigma$ 112 1	58 8	19 39
$\mu$ 11 21	5 88	1 939
$\sigma$ 2 56	1 87	0 413

<sup>a</sup> The ratio of the volume of water collected in fifteen minutes from normal skin to aluminum sulfate treated skin in microliters

TABLE III.—A COMPARISON OF THE AMOUNT OF WATER COLLECTED FROM A LIMITED AREA OF SKIN TREATED WITH ANTIPERSPIRANT BASES

Normal Skin or Base	Microliters $\text{H}_2\text{O}^a$		Ratio <sup>b</sup>
	Normal or Base	Aluminum Sulfate	
Normal	13 0	8 0	1 625
Stick	13 3	8 3	1 602
Cream	12 0	8 0	1 500
Lotion	12 0	7 5	1 600

<sup>a</sup> Each value represents the mean of three experiments on the same limited area

<sup>b</sup> The ratio of the means of normal skin or areas treated with the three bases to adjacent aluminum sulfate treated areas

and aluminum sulfate. One test was run once a day for three days on each area, an area being a treated or a controlled portion of skin adjacent to each other.

**Constant Values.**—It was noted that as each of the products was applied on successive days, the variation in the amount of water perspired tended to decrease, and that after a certain length of time (four to six days for the products, three to four days for aluminum sulfate) a fairly constant volume and ratio were obtained (see Table IV). Variations in the total volume changed with the temperature and humidity, but the ratio remained fairly constant.

**Commercial Antiperspirant Products.**—Six commercial and one research product were compared

TABLE IV.—A COMPARISON OF THE PERSPIRATION REDUCING PROPERTIES OF ALUMINUM SULFATE CREAM WITH 24% w/w ALUMINUM SULFATE SOLUTION OVER A LIMITED AREA OF SKIN

Days	Microliter of $\text{H}_2\text{O}$ , $\mu$ L <sup>a</sup>		Ratio <sup>b</sup>
	Aluminum Sulfamate	Aluminum Sulfate	
1	6.1	4.6	1.34
2	5.9	4.2	1.41
3	7.3	5.0	1.45
$\Sigma$	19.3	13.8	8.40
$\mu$	6.42	4.60	1.400
$\sigma$	0.682	0.438	0.101

<sup>a</sup> Each value represents the mean amount of water collected from the same area twice daily, a constant volume of water was collected after four days

<sup>b</sup> The ratio of the means of aluminum sulfamate- to aluminum sulfate treated skin



TABLE V.—A COMPARISON OF THE AMOUNT OF SENSIBLE PERSPIRATION COLLECTED FROM LIMITED SKIN AREAS TREATED WITH ANTIPERSPIRANT PREPARATIONS AND 24% w/w  $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$  SOLUTION

Preparations	Antiperspirant Preparations	Al <sub>2</sub> (SO <sub>4</sub> ) <sub>3</sub> · 18H <sub>2</sub> O Soln.	Mean Ratio
	Mean Response <sup>a</sup> μL. <sup>b</sup>	Mean Response <sup>a</sup> μL. <sup>b</sup>	
A <sup>c</sup>	8.4	5.9	1.52 <sup>d</sup>
B	8.8	5.3	1.68
C	9.2	4.9	2.09
D <sup>e</sup>	5.6	4.6	1.21
E	6.7	4.9	1.40
F	7.4	4.9	1.52
G	6.4	4.6	1.40

<sup>a</sup> A mean of ten determinations on five consecutive days were used to calculate the mean response of each antiperspirant preparation tested.

<sup>b</sup> The microliters of water collected in fifteen minutes were calculated using the standard curves for electrolytic cells R<sub>1</sub> and R<sub>2</sub>.

<sup>c</sup> Active ingredients: A, aluminum hydroxide gel; B, aluminum chloride and formaldehyde; C, aluminum chlorohydroxide complex, aluminum chloride, and formaldehyde; D, aluminum methionate; E, sodium aluminum lactate and hexachlorophene; F, aluminum chlorohydrates; G, aluminum sulfamate.

<sup>d</sup> This ratio indicates that 1.52 times as much sensible perspiration was collected from the skin areas treated with antiperspirant preparation A as that from the aluminum sulfate-treated area.

<sup>e</sup> A preparation prepared by J. E. Haberle, M.S. thesis, Purdue University, Lafayette, Ind., June, 1958.

with the aluminum sulfate solution. The antiperspirants were applied to the chosen areas until constant volumes were obtained, and then the experiment was continued for another three days. Table IV is an example of the type of data obtained. The active ingredient was aluminum sulfamate in a cream base.

Table V shows the mean volumes of water collected when the six commercial products and the one research product (aluminum methionate) were compared with 24% w/w aluminum sulfate solution. The volume ratio indicates the ability of the anti-

perspirant to reduce perspiration flow as compared with 24% w/w aluminum sulfate solution. The results indicate that the research compound, aluminum methionate, was the most effective of the products studied to reduce sensible perspiration flow.

## SUMMARY AND CONCLUSIONS

1. A continuous recording *in vivo* method of measuring sensible perspiration was used to evaluate the relative perspiration properties of six commercial and one research antiperspirant preparation in comparison with 24% w/w  $\text{Al}_2(\text{SO}_4)_3 \cdot 18\text{H}_2\text{O}$  solution. The results indicated that aluminum methionate was the most effective agent for reducing perspiration flow from the treated area.

2. The method was also used to evaluate three different types of antiperspirant bases: the stick, the cream, and the lotion. The results when compared with normal skin indicated that the bases had no effect on the antiperspirant action of the preparations.

## REFERENCES

- (1) O'Malley, W. J., and Christian, J. E., *THIS JOURNAL*, **49**, 398(1960).
- (2) Govett, T., and de Navarre, M. G., *Am. Perfumer Essent. Oil Rev.*, **51**, 365(1947).
- (3) Christian, J. E., and Jenkins, G. L., *THIS JOURNAL*, **39**, 663(1950).
- (4) Uarakama, C., and Christian, J. E., *ibid.*, **42**, 179(1953).
- (5) Collins, S. F., and Christian, J. E., *ibid.*, **47**, 25(1958).
- (6) Fredell, W. G., *Drug & Cosmetic Ind.*, **69**, 41(1951).
- (7) Richardson, S. L., and Meigs, B. V., *J. Soc. Cosmetic Chemists*, **2**, 308(1950).
- (8) Marchositto, R., Ph.D. thesis, Purdue University, Lafayette, Ind., Jan. 1956.
- (9) Palmes, E. D., *Rev. Sci. Instr.*, **19**, 711(1948).
- (10) Hill, L., and Hargood-Ash, *Proc. Phys. Soc. London*, **33**, 169(1921).
- (11) Staey, R. W., et al., "Essentials of Biological and Medical Physics," McGraw-Hill Book Co., New York, N. Y., 1955, p. 153.
- (12) Randall, W. C., *J. Clin. Invest.*, **25**, 761(1946).

## Determination of Vitamin A: Errata\*

Sir:

In the paper titled "A Simplified Procedure for the Determination of Vitamin A" (1), the first footnote carried under Table I is incorrect. This should read as follows:

$$f(\text{MS}) = \frac{A_{\text{corr.}}}{A_{\text{un-corr.}}}$$

$$A_{\text{corr.}} = 6.815(A_{725}) - 2.555(A_{725}) - 4.260(A_{770})$$

$$A_{\text{un-corr.}} = A_{770}$$

Furthermore, in all Tables,  $F(\text{MS})$  should read  $f(\text{MS})$ .

(1) Napoli, J. A., Senkowski, B. Z., and Metchane, A. F., *THIS JOURNAL*, **48**, 611(1959).

E. G. E. SHAFER  
Hoffmann-La Roche, Inc.  
Nutley, New Jersey

\* Received March 4, 1960

# Notes

## A Note on Esters of $\beta$ -Phenyl- $\alpha$ -benzyloximinopropionic Acid\*

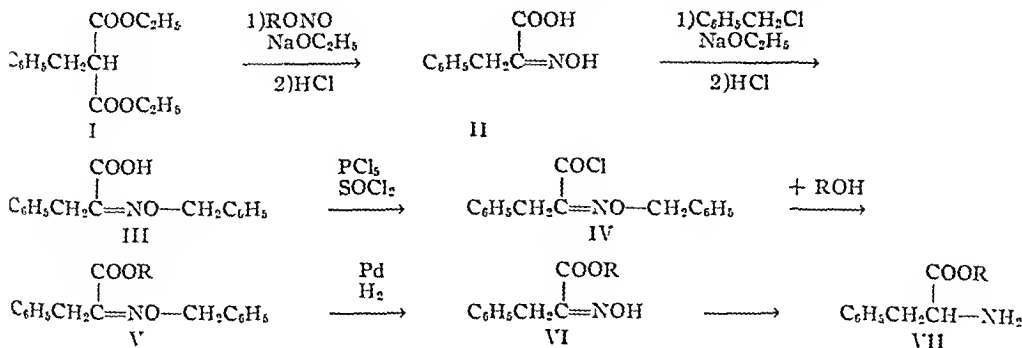
By LOUIS GASS† and JOHN W. MARTIN, Jr.

Selected esters were prepared for the purpose of studying the debenzoylation reactions when the alcohol portion of the esters was modified. A method is given to obtain esters of oximino acids. Except for phenolic derivatives, the esters were fairly easily prepared from the alcohol and the acid chloride.

IN A REPORTED synthesis of amides of  $\alpha$ -amino acids (1) the  $\alpha$ -benzyloximino acid is the key intermediate. In the present investigation esters of  $\alpha$ -benzyloximino acid have been synthesized (V). These are intermediates necessary in the study of the hydrogenolysis and reduction reactions leading to esters of  $\alpha$ -amino acids (VII). The entire sequence of reactions is shown below. Compounds of type VI and VII are not reported in this paper.

The esters thus prepared represent different categories which can be studied chemically and pharmacologically. From preliminary experiments with the debenzoylation reaction (V to VI) it appears that the rate of debenzoylation is greatly influenced by the type of derivative.

Compound I, a substituted malonic ester, was prepared by a standard procedure (2). The nitration of I was done according to the method of Waters and Hartung (3), with a slight modification using isopropyl nitrite rather than the butyl nitrite. Compound II was obtained in good yields. Efforts to prepare the acid chloride directly proved unsuccessful (4) so compounds III and IV were prepared by methods reported by Hartung and co-workers (1-5). Compound V, heretofore not prepared by this route, involved the reaction of the acid chloride directly with the alcohol in absolute ether.



\* Received April 13, 1956, from Butler University, College of Pharmacy, Indianapolis 7, Ind.

Abstracted from a thesis submitted as partial fulfillment of the requirement for the degree of Master of Science, Butler University, College of Pharmacy, Indianapolis, Ind.

Presented to the Scientific Section, A. P. A., Detroit meeting, April 1956.

† Present address: Whitehall Laboratories, Hammon-ton, N. J.

## EXPERIMENTAL

All melting points reported were obtained by use of a Fisher-Johns melting point apparatus.

**Amyl  $\beta$ -Phenyl- $\alpha$ -benzyloximinopropionate.**—Four and four-tenths grams (0.05 M) of freshly distilled amyl alcohol was placed in a three-necked round-bottom flask to which was added 60 ml. of dry ether. The flask was placed in an ice bath and, with mechanical stirring, an equimolar portion of a benzene solution of the acid chloride was dropped in slowly. After the complete addition of the acid chloride stirring was continued for ten minutes. The yellow solution was washed with 100 ml. of a saturated solution of sodium bicarbonate and then dried over calcium chloride for forty-eight hours. After removal of the ether under vacuum there remained 16.9 Gm. (quantitative) of a yellow viscous oil. Upon attempted vacuum distillation the product decomposed.

*Anal.*—Calcd. for  $\text{C}_{21}\text{H}_{25}\text{NO}_3$ : N, 4.13; sapon. equiv., 339. Found: N, 3.95, 3.91; sapon. equiv., 338.2, 339.6

**Benzyl  $\beta$ -Phenyl- $\alpha$ -benzyloximinopropionate.**—Ten and eight-tenths grams (0.1M) of freshly distilled benzyl alcohol was dissolved in 50 ml. of dry ether in a small beaker cooled in an ice bath. To this was added slowly, with rapid stirring, a benzene solution of an equimolar amount of acid chloride, prepared from  $\beta$ -phenyl- $\alpha$ -benzyloximino propionic acid. After standing for about ten minutes in the ice bath the ether was removed and the remaining solid was recrystallized twice from alcohol with the addition of Nuchar. After cooling, the crystals were collected. After drying *in vacuo* over  $\text{P}_2\text{O}_5$  the product weighed 31.5 Gm. (87.7%), m. p. 79–80°.

*Anal.*—Calcd. for  $\text{C}_{21}\text{H}_{23}\text{NO}_3$ : N 3.90; sapon. equiv., 359. Found: N, 3.78, 3.72; sapon. equiv., 361, 358.1, 358.8.

**Cyclohexyl  $\beta$ -Phenyl- $\alpha$ -benzyloximinopropionate.**—Essentially the same procedure was used as for the amyl derivative. From 5 Gm. (0.05M) of

cyclohexanol and an equimolar quantity of the acid chloride, 15.5 Gm (88%) of liquid was obtained. The boiling point by the semimicro method was 189°, with decomposition.

*Anal*—Calcd for  $C_{22}H_{25}NO_3$ : N 3.99, sapon equiv, 351. Found: N, 3.89, 3.83, sapon equiv, 350.7, 351.3.

**Naphthyl  $\beta$ -Phenyl- $\alpha$ -benzyloximinopropionate.**—Essentially the same procedure was used as for the amyl ester. From 7.2 Gm (0.05 M) of purified  $\alpha$ -naphthol and an equimolar amount of the acid chloride, 16.5 Gm (83.5%) of a viscous oil was collected. Naphtha was used as the solvent. The oil decomposed upon attempted distillation.

*Anal*—Calcd for  $C_{26}H_{21}NO_3$ : N, 3.54, sapon equiv 395. Found: N, 3.36, 3.25, sapon equiv, 389.8, 390.6, 389.6.

**Furfuryl  $\beta$ -Phenyl- $\alpha$ -benzyloximinopropionate.**—Nine and eight-tenths grams (0.1 M) of freshly distilled furfuryl alcohol was placed in a beaker cooled in an ice bath. Sixty milliliters of anhydrous ether and 7.9 Gm (0.01 M) of freshly distilled pyridine were added and then, with rapid stirring, an equimolar portion of a benzene solution of the acid chloride. There was an immediate bubbling and the solution turned black. This mixture was allowed to stand in the ice bath for fifteen minutes

and then was washed several times with water to remove the pyridine hydrochloride. After evaporation of the ether and long standing the mixture crystallized. The large crystals were purified by recrystallization from alcohol and water. After drying *in vacuo* over  $P_2O_5$  the product weighed 21.5 Gm (61.6%), m p 38–39°.

*Anal*—Calcd for  $C_{21}H_{19}NO_4$ : N 4.01, sapon equiv, 349. Found: N, 3.85, 3.90, 3.84, sapon equiv, 347.5, 347.6.

## SUMMARY

Five selected esters of  $\beta$ -phenyl- $\alpha$ -benzyloximinopropionic acid have been prepared and characterized.

## REFERENCES

- (1) Martin, J. W., and Hartung, W. H., *J. Org. Chem.* 19, 358 (1954).
- (2) Shirley, D. A., "Preparation of Organic Intermediates," John Wiley & Sons, Inc., New York, N. Y., 1951, 147.
- (3) Waters, K. L., and Hartung, W. H., *J. Org. Chem.* 12, 469 (1947).
- (4) Weaver, W. E., and Hartung, W. H., *ibid.* 15, 741 (1950).
- (5) Barry, R. H., and Hartung, W. H., *ibid.* 12, 400 (1947).

# A Note on the Synthesis of C-14 Carboxyl Salicylic Acid by the Halogen-Metal Interconversion Reaction\*

By WILLIAM F. BOUSQUET and JOHN E. CHRISTIAN

IN ORDER TO CARRY OUT a study of the central nervous system distribution and metabolism of C-14 carbonyl salicylamide in the rat (1), it was necessary to prepare C-14 carboxyl labeled salicylic acid for use in synthesizing the amide.

The preparation of C-14 carboxyl salicylic acid has been described in the literature by a number of authors. Mandel and Smith (2), Schayer (3), and Borst and Christian (4) have described the synthesis of this labeled compound by the Kolbe-Schmitt reaction. Medenwald and Haberland (5) have recently described a synthesis of carboxyl labeled salicylic acid by the Grignard reaction.

The method of synthesis chosen was the halogen-

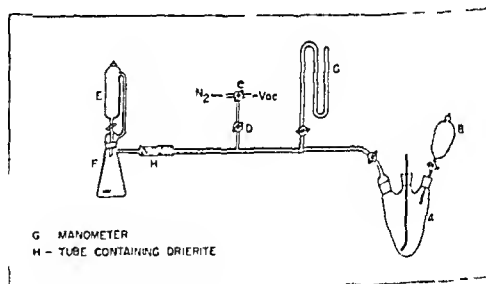
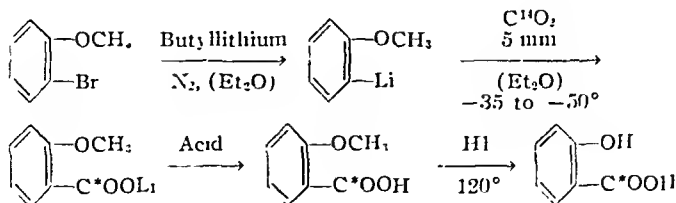


Fig. 1—Gaseous carbonation apparatus



metal interconversion reaction on *o*-bromoisotonic followed by carbonation of the lithium salt *in situ* with  $\text{C}^{14}\text{O}_2$ . The C-14 carboxyl *o*-methoxybenzoic acid so prepared was then treated with HI to liberate salicylic acid.

The primary advantages of this method in the synthesis of C-14 carboxyl salicylic acid are that it is not necessary to work with  $\text{C}^{14}\text{O}_2$  under heat and pressure, and specificity of labeling is attained. Furthermore, C-14 carboxyl *p*-hydroxybenzoic acid is not a by-product in the reaction as is the case with the Kolbe-Schmitt procedure.

\* Received August 21, 1959, from the Bionucleonics Department, Purdue University, Lafayette, Ind.

The synthesis was sealed to give a theoretical yield of 1.0 Gm. (0.00725 M) of salicylic acid. The apparatus used in the carbonation, similar to that of Dauben, *et al.* (6), and Rockerbie (7), is shown in Fig. 1. Seven runs were made without radioactivity in order to perfect the synthesis. Four additional runs were made using small quantities (e. g., 100–200  $\mu$ c.) of radioactivity. Yields of purified salicylic acid ranged from 6 to 43% of the theoretical. The procedure described below is that followed in the final run with 25 mc. of C-14 activity.

### PROCEDURE

The *n*-butyllithium was prepared by the procedure of Gilman, *et al.* (8), and assayed before use by the method of Gilman and Haubein (9). All glassware used in the synthesis was cleaned with chromic acid-sulfuric acid cleaning solution, rinsed with distilled water, oven-baked, and flushed with dry nitrogen immediately before use.

A measured amount of the *n*-butyllithium solution in ether corresponding to 20 mM was transferred to reaction flask (A) while the system was being flushed with dry nitrogen. The nitrogen was turned off, stopcock (D) closed, and addition funnel (B) containing 1.95 Gm. (1.3 ml., 10 mM) of *o*-bromoanisole in 5 ml. of anhydrous ether set in place. A Dewar flask containing acetone-dry ice was placed under the reaction flask. The *o*-bromoanisole was rapidly added to the butyllithium solution and the reaction allowed to proceed with gentle stirring for fifteen minutes. The stirrer was shut off and the acetone-dry ice bath replaced with one of liquid nitrogen. The contents of the reaction flask were frozen solid and the system evacuated to about 3 mm. The acetone-dry ice bath was again set in place. When the pressure in the system remained constant at from 5 to 8 mm. the stirrer was set at high speed and concentrated sulfuric acid added from addition funnel (E) to the  $\text{BaC}^{14}\text{O}_3$  in generator flask (F).

The magnetic stirrer in the generator flask was set in motion and stirring continued until gas evolution appeared complete. After an initial rise, the pressure in the system fell rapidly as the carbon dioxide was taken up in the reaction mixture. Stirring was continued until the pressure remained constant. The contents of the reaction flask were alternately frozen solid and thawed twice to insure maximum uptake of  $\text{C}^{14}\text{O}_2$ .

The male stopper in addition funnel (B) was replaced with a T/S 24/40 connecting tube, and a 12-inch length of rubber tubing used to connect this to a barium hydroxide absorption train. The system was then opened to allow nitrogen to enter and bring the system to atmospheric pressure. Stopcock (C) was opened and nitrogen flushed through the system into the absorption train to allow collection of unreacted  $\text{C}^{14}\text{O}_2$ . Flushing was continued for twenty-five minutes. The nitrogen was then shut off, the system closed, and the absorption train removed.

Ten milliliters of a 20% solution of hydrochloric acid was placed in addition funnel (B) and added, with rapid stirring, to the reaction mixture. The contents of flask (A) were then transferred to a 125-ml. separatory funnel. The flask was washed with two 10-ml. portions of ether and distilled water, and

the washings added to the separatory funnel. The aqueous layer was drawn off and set aside. The ether layer was extracted with three 5-ml. portions of 10% sodium hydroxide solution. The aqueous layer was extracted with three 5-ml. portions of ether, and this was extracted with two 5-ml. portions of 10% sodium hydroxide. The sodium hydroxide extracts were pooled, set in an ice bath, and concentrated hydrochloric acid added dropwise to precipitate C-14 carboxyl *o*-methoxybenzoic acid. The acid was collected by suction filtration and oven dried at 60°.

The dried crystals obtained above were transferred to a 50-ml. round-bottom flask to which was added 5 ml. of 47% hydriodic acid (sp. gr. 1.50). The flask was fitted with a reflux condenser, set in an oil bath, and the temperature raised and maintained at 120–125° for thirty minutes. Upon cooling, long white needles of C-14 carboxyl salicylic acid precipitated. The mixture was allowed to stand overnight and the salicylic acid then removed by suction filtration. The crystals were dried at 110° and allowed to stand exposed to air for forty-eight hours to remove any adsorbed iodine.

Pure salicylic acid recovered amounted to 67.0 mg. with a specific activity of  $5.59 \times 10^7$  dpm/mg. An additional 13.0 mg. of material was recovered, having a specific activity of  $3.52 \times 10^7$  dpm/mg. The chemical yield of purified C-14 carboxyl salicylic acid was 6.7%. Radiochemical yield<sup>1</sup> was 8.2%. A melting point was not taken on this material due to its high activity. However, melting points were determined on the salicylic acid prepared in the dry runs and these were in agreement with published values for salicylic acid.

Chemical and radiochemical purities of the labeled compound were determined using a combination of paper chromatography and autoradiography. Chromatograms were prepared using the ascending technique with a solvent system of benzene:glacial acetic acid:water (4:4:2). Whatman No. 1 paper was used. Color development was attained by spraying with ferric nitrate solution. The  $R_f$  value of the synthesized compound was found to be identical with that for reference salicylic acid.

Preparation of an autoradiogram from the chromatogram using Eastman-Kodak "No-Screen" X-ray film showed the presence of but one spot corresponding to the synthesized material. No radiochemical contaminants were present.

### REFERENCES

- (1) Bousquet, W. F., and Christian, J. E., *THIS JOURNAL*, **49**, 389 (1960).
- (2) Mandel, H. G., and Smith, P. K., *ibid.*, **39**, 479 (1950).
- (3) Schayer, R., *Arch. Biochem.*, **28**, 371 (1950).
- (4) Borst, W. R., and Christian, J. E., *THIS JOURNAL*, **45**, 23 (1956).
- (5) Medenwald, H., and Haberland, G. L., *Z. physiol. Chem. Hoppe-Seyler's*, **306**, 229 (1957).
- (6) Dauben, W. G., Reid, J. C., and Yankwich, P. E., *Anal. Chem.*, **19**, 828 (1947).
- (7) Rockerbie, R. A., M.S. Thesis, Purdue University Library (1954).
- (8) Gilman, H., Beel, J. A., Brannen, C. G., Bullock, M. W., Dunn, G. E., and Miller, L. S., *J. Am. Chem. Soc.*, **71**, 1499 (1949).
- (9) Gilman, H., and Haubein, A. H., *ibid.*, **66**, 1515 (1944).

<sup>1</sup> The specific activity of the synthesized material was determined by dissolving 0.5065 mg., accurately weighed, in 50 ml. of toluene containing 50 mg. of carrier salicylic acid. The activity of 100  $\mu$ l. aliquots of this solution was determined using the Tri-Carb liquid scintillation spectrometer. Correction for counter efficiency was made using a National Bureau of Standards C-14 carboxyl benzoic acid beta ray standard.

# A Note on Improvement of Separations in Paper Partition Chromatography<sup>a</sup>

By JERE E GOYAN

FOR MANY YEARS it has been recognized that the separation of two substances by multiple fractional extraction is greatest when the volume ratio of the two solvents ( $V_1/V_2$ ) is equal to  $\sqrt{1/\alpha_1 \alpha_2}$ , where  $\alpha_1$  and  $\alpha_2$  are the partition coefficients for the two components being separated ( $\alpha_1 = \text{mg 1 per cc of 1/mg 1 per cc of 2}$ ) (1). The same principles are involved in paper chromatography, which may be approximated as a series of extractions, and it might be expected that a similar ratio would be important in partition chromatography. In the course of a project on the separation of barbiturate isomers such a relationship was found.

The operational definition of  $R_f$  may be stated as

$$R_f = A_M / (A_M + \alpha A_S) \quad (\text{Eq 1})$$

(2) where  $A_M$  is the cross sectional area of the mobile phase,  $A_S$  is the cross sectional area of the stationary phase, and  $\alpha$  is the partition coefficient for the component between the mobile and stationary phase.

Solving Eq 1 for  $\alpha$  one obtains

$$\alpha = \frac{A_M}{A_S} \left[ \frac{1}{R_f} - 1 \right] \quad (\text{Eq 2})$$

then defining  $\beta = \alpha_1/\alpha_2$

$$\beta = R_{f2}(1 - R_{f1})/R_{f1}(1 - R_{f2}) \quad (\text{Eq 3})$$

The most desirable separation for two components is when  $R_f - R_{f1}$  is a maximum. Solving Eq 3 for  $R_{f2}$  and subtracting  $R_{f1}$  from both sides of the equation, one obtains

$$\gamma \equiv R_{f2} - R_{f1} = \beta R_{f1} / (1 - R_{f1} + \beta R_{f1}) - R_{f1} \quad (\text{Eq 4})$$

Then setting the first derivative of  $\gamma$  with respect to  $R_{f1}$  equal to zero and solving for  $R_{f1}$  the following equation is found

$$R_{f1} = 1/(1 + \sqrt{\beta}) \quad (\text{Eq 5})$$

Rewriting Eq 1 in terms of volumes gives (3)

$$R_{f1} = V_M / (V_M + \alpha V_S) \quad (\text{Eq 6})$$

Then equating 5 and 6 and solving one obtains

$$V_M/V_S = \sqrt{1/\alpha_1 \alpha_2} \quad (\text{Eq 7})$$

which is the usual equation for maximizing separation in multiple fractional extraction procedures. This shows the correlation which is to be expected if paper partition chromatography can be approximated as a multiple extraction process.

Unfortunately, adjustment of the volumes of the stationary and mobile phases is difficult. It is possible to try several different papers and to choose the one giving the best separation. There is, however, another method of adjustment of  $R_{f1}$  in cases where

three component systems are used. When  $\alpha$  changes are made in phase concentrations  $\beta$  changes very little, since both values of  $\alpha$  change in the same direction. Thus it becomes possible to change  $\beta$  considerably while having only secondary effect on  $\beta$ .

These principles may be illustrated by some experimental data on the amino acids, glycine and alanine. Block, *et al* (4), give data for these two amino acids in two different butanol-acetic acid-water systems, these data contributing the first two rows of Table I.

TABLE I —  $R_f$  VALUES OF GLYCINE AND ALANINE IN BUTANOL-ACETIC ACID-WATER MIXTURES

Acetic Acid % v/v	Glycine	Alanine	$\beta$
5 <sup>a</sup>	0.03	0.05	1.9
10.6 <sup>a</sup>	0.05	0.09	1.7
12.5	0.25	0.33	1.5
13.0	0.33	0.44	1.6

<sup>a</sup> Data from Block *et al* (4)

At first glance it might appear impossible to separate the two amino acids with this solvent system. However, solving for  $\beta$  using Eq 3, gives values of 1.9 and 1.7. Solving for  $R_{f1}$  in Eq 5 (using  $\alpha = 1$ ) gives a value of 0.43. Thus if would expect the better separations could be expected if  $R_{f1}$  is increased. In order to test this the following experiment was undertaken. The  $R_f$  should increase if the mobile phases become a better solvent for an amino acid, therefore the amount of acetic acid was increased. Values of 12.5 and 13.0% v/v acetic acid (equal parts of butanol and water) were chosen since greater percentages lead to a one phase system. These were run on Whatman No. 1 filter strips with descending formation. Table I shows the results of these experiments. Thus it was possible to get reasonable separations with a system which originally did not look promising. Parallel experiments using Whatman No. 4 and 54 paper gave  $R_{f1}$  values which were smaller and the separation was correspondingly less.

TABLE II —  $R_f$  VALUES OF PENTOBARBITAL (I) AND ITS 1-ETHYLPROPYL ISOMER (II) IN BENZENE-ETHANOL-ACETIC ACID MIXTURES

Ethanol % v/v	I	II	$\beta$
25	0.90	0.91	1.1
15	0.85	0.88	1.1
10	0.77	0.81	1.3
0	0.71	0.78	1.4

In the experiments, with pentobarbital and its 1-ethylpropyl isomer, using 1 benzene-10% acetic acid-ethanol system and reversed phase (60% treated with 5% SF 96 (300) silicone fluid, G. E. Electric, in *n*-pentane and dried in an oven for three minutes at 150°) descending development it

<sup>a</sup> Received February 15, 1960, from the College of Pharmacy, University of Michigan, Ann Arbor.  
This research was supported by a grant from the Hersey H. Rackham School of Graduate Studies, The University of Michigan.

found that  $R_{f1}$  was too large and that decreasing the ethanol concentration led to an improvement in the separation, as shown in Table II.

The general method may be extended, of course, to larger numbers of substances. Analysis of preliminary data in this manner should make it possible to make a more rational decision as to means of improving desired separations.

## REFERENCES

- (1) Bush, M T, and Densen, P M, *Anal Chem*, **20**, 121(1948)
- (2) Lederer, E, and Lederer, M, "Chromatography: A Review of Principles and Applications" Elsevier Publishing Corp, New York, N Y, 1954, p 104
- (3) Cassidy, H G, "Fundamentals of Chromatography," Interscience Publishers, Inc, New York, N Y, 1957, p 60
- (4) Block, R C, Durrum, E L, and Zweig, G, "A Manual of Paper Chromatography and Paper Electrophoresis" Academic Press Inc, New York, N Y, 1958, p 148

---

## Book Notices

---

*New and Nonofficial Drugs 1960* Evaluated by A M A Council on Drugs Medical Dept, J B Lippincott Co, East Washington Square, Philadelphia 5, Pa, 1960 xviii + 768 pp 12 5 x 19 cm Price \$3 35

The new subtitle for the 1960 edition of N N D reads: "An annual compilation of available information on drugs, including their therapeutic, prophylactic, and diagnostic status, as evaluated by the Council on Drugs of the American Medical Association." Its scope comprises agents proposed for use whether or not their usefulness has been definitely established. The A M A thus attempts to inform the physician about the values and dangers of new drugs as soon as responsible and authoritative opinions can be obtained. Forty-five new monographs have been added in N N D 1960. It is to be hoped that the excellent work of the Council on Drugs could be broadened to publish its evaluations of new drugs even sooner, without sacrificing its unbiased, thorough approach. This might discourage the reading of hastily published opinions by other less qualified medical "experts."

*The Merck Index of Chemicals and Drugs* 7th ed Edited by PAUL G STECHER, M J FINKEL, and O H. SEGUMUND. Merck & Co, Inc, Rahway, N J, 1960 xi + 1641 pp 18 x 25 cm. Price \$12

This exceptionally useful and reliable reference book has 400 pages more than the 6th ed of "The Merck Index," which was reviewed in THIS JOURNAL, 41, 339(June 1952). The increase in page size also is significant, from 16 x 23 5 cm in the 6th to 18 x 25 cm in the 7th ed. The new edition includes nearly 10,000 descriptions of individual substances, more than 3,300 structural formulas, and about 30,000 names of chemicals and drugs in a separate, alphabetically arranged and cross-indexed listing. The separate listing should be utilized, rather than leafing through the extensive encyclopedic text, to find any substance or compound by its systematic-chemical, generic, common, brand, or trade name.

A special section lists more than 400 organic "name" reactions with a description and structural representation of each. Additional information includes a list of prescription notations and a table of isotonic solutions of drugs.

*Pharmacopoea Internationalis, 1st Edition Supplement* World Health Organization, Geneva, 1959. Official distribution agent for the U S Columbia University Press, 2960 Broadway, New York 27, N Y xx + 224 pp 16 x 24 cm Price \$5

This supplementary volume containing 94 additional monographs and 17 appendices completes the first edition of the International Pharmacopoeia (Ph I). Volumes I and II were reviewed in THIS JOURNAL, 41, 222(1952) and 45, 434(1956). The Ph I is a collection of recommended specifications which are not intended to have legal status, as such, in any country but which can serve as a basis for the establishment of national standards. Appendix 7, titled "Preparation of Isotonic Solutions," includes graphs showing the amount of sodium chloride (or potassium nitrate) to be added to solutions of 67 drugs to obtain isotonicity. A composite index for Volumes I and II and the Supplement is appended. A table comparing the titles and strength standards of drugs appearing in both Ph I and N F XI will be included in the General Information section of N F XI.

*Year Book of Drug Therapy 1959-1960* Series Edited by HARRY BECKMAN. The Year Book Publishers, Inc, 200 East Illinois St, Chicago 11, Ill, 1960 lxxx + 570 pp 13 x 19 5 cm Price \$8 50

This edition of the Year Book continues Dr Beckman's excellent coverage and condensation of the medical literature. He has added a section in which he gives brief opinions on new drugs for which FDA new drug applications became effective during the period from September 1958 to September 1959.

*American Drug Index 1960*. By CHARLES O WILSON and TONY EVERETT JONES. J B Lippincott Co, East Washington Square, Philadelphia 5, Pa, 1960 712 pp 13 5 x 20 5 cm Price \$5 75

The 1960 edition of "American Drug Index" brings up to date this very useful reference of pharmaceutical chemicals and preparations. The original format and concise style of coverage is unchanged.

# The United States Pharmacopœial Convention, Inc.

## ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the  
year ended December 31, 1959

### STATEMENT OF INCOME AND EXPENSE

<b>Income:</b>			
Sales of Pharmacopœias:			
Collections.....		\$25,133.25	
Add—Increase in accounts receivable.....		454.90	
			\$ 25,588.15
Sales of reference standards:			
Collections.....		\$49,742.00	
Add—Increase in accounts receivable, and adjustment o. prior years' collections.....		2,323.15	
			52,065.15
Interest on investments, less amortization of bond premiums of \$22.65.....			6,839.85
Interest on savings deposits.....			6,012.41
Use of text by others.....			25.00
Miscellaneous.....			121.31
			<u>\$ 90,651.92</u>
<b>Expense:</b>			
Revision.....		\$86,330.41	
Less—Increase in inventory.....		340.76	
			\$ 85,989.65
Administration.....			8,783.67
Publications.....		\$27,031.43	
Add—Decrease in inventory.....		10,543.02	
			37,574.45
Headquarters.....			\$ 5,658.97
Add—Depreciation expense			
Building.....		\$2,430.77	
Furnishings and equipment.....		4,208.99	6,639.76
			12,298.73
Convention.....			4,421.75
			<u>\$119,071.21</u>
Excess of Expense over Income.....			<u>\$ 58,419.31</u>

### STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

<b>Receipts:</b>			
Sales of pharmacopœias.....			\$ 25,133.25
Sales of reference standards.....			49,742.00
Interest on investments.....			6,862.50
Interest on savings deposits.....			6,012.41
Use of text by others.....			25.00
Miscellaneous.....			121.31
Transfer from savings accounts.....			18,009.00
			<u>\$106,579.92</u>

	Revision	Adminis- tration	Publications	Head- quarters	Convention	Furnish- ings and Equip- ment	Total
<b>Disbursements:</b>							
Printing and binding.....			\$27,031.43				\$ 27,031.43
Salaries.....	\$57,209.72	\$3,590.20		\$1,791.20	\$ 390.20		61,681.32
Meetings.....	2,613.77	1,455.42			3,596.78		7,665.97
Supplies.....	1,113.52	46.41			210.27		1,470.20
Postage and telegrams.....	1,709.02	36.11			191.50		1,936.63
Utilities.....				2,328.71			2,328.71
Repairs and maintenance.....				1,351.03			1,351.03
General.....	23,681.38*	1,655.50					25,336.88
Insurance and taxes.....				185.00			185.00
Furnishings and equipment.....						\$178.75	178.75
	<u>\$86,330.41</u>	<u>\$5,783.67</u>	<u>\$27,031.43</u>	<u>\$5,658.97</u>	<u>\$1,121.75</u>	<u>\$178.75</u>	<u>\$132,704.98</u>

* Research Assistants.....	\$ 4,166
Reference Standards supplies.....	5,137
Retirement and Social Security.....	11,868
Promotion.....	103
Miscellaneous.....	2,410
	<u>\$23,684</u>

*Scientific Edition*  
**JOURNAL OF THE  
AMERICAN PHARMACEUTICAL  
ASSOCIATION**

VOLUME 49

JULY 1960

NUMBER 7

## The Avidity of Salicylic, Gentisic, and Salicyluric Acids for Heavy Metal Cations\*

By JOSEPH PECCI† and WILLIAM O. FOYE

Potentiometric titrations were carried out with the title compounds in the presence of various heavy metal cations. Evidence was provided by this method that  $\text{Cu}^{++}$ ,  $\text{Fe}^{+++}$ , and  $\text{Al}^{+++}$  ions were capable of forming stable chelates with these acids. No evidence of chelation was found with  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ , or  $\text{Ag}^{+}$  ions, although chelates of low stability would not be detected by this procedure. The avidities of these acids for metal ions were recorded as stability constants, and their magnitude indicates that the chelates should be capable of existence in the animal cell.

IT HAS BEEN suggested that salicylates may exert some, if not all, of their biological effects through their ability to chelate the ions of metals (1). The nonchelating *meta*- and *para*-hydroxybenzoic acids show none of the classical actions of the salicylates (2). It was therefore thought enlightening to examine salicylic acid and its major metabolites, salicyluric and gentisic acids, in regard to the heavy metal cations that they are capable of chelating, and also to know quantitatively the avidity with which these salicylates bind metal ions.

Potentiometric titration appeared to be a suitable method for obtaining the desired information. Examination of the curves produced would indicate whether or not chelation had occurred, and stability constants could be calculated from the pH measurements. Mathematical treatment of the results was that derived by Albert (3, 4) for chelating agents having two ionizing groups. Babko (5, 6, 7) has previously obtained stability constants for salicylic acid with ferric, cupric,

and aluminum ions using a spectrophotometric procedure based on the method of Job (8).

### METHODS

**Materials.**—The metallic ions were used in the form of the following salts: Fisher certified  $\text{CuCl}_2$ , Baker analyzed reagents  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{Ca}(\text{CH}_3\text{CO}_2)_2 \cdot \text{H}_2\text{O}$ , and  $\text{AgNO}_3$ . The *o*-hydroxybenzoic acid used was Fisher certified grade, the *p*-hydroxybenzoic acid obtained from Eastman Organic Chemicals, and the gentisic acid obtained from Matheson, Coleman and Bell. The salicyluric acid was prepared by the procedure of Hanzlik (9), m. p. 168–170°.

Carbonate-free 0.100 *N* sodium hydroxide was prepared from a filtered saturated solution of sodium hydroxide U. S. P. The stock solution was appropriately diluted with freshly boiled-out and cooled distilled water, and standardized.

**Titration.**—Exactly 50.0 ml. of a 0.01 *M* solution of salicylic acid or derivative was titrated with 0.100 *N* sodium hydroxide, first in the absence of metal ions and then in the presence of 0.00025 mole of a divalent metal salt or 0.000165 mole of a trivalent metal salt (one equivalent). Thus the molar concentration was 0.005 *M* for divalent ions and 0.0033 *M* for trivalent ions, and the molar ratio of salicylic acid to metal was 2:1 for divalent and 3:1 for trivalent ions. The total volume of the solution being titrated was therefore 50 ml., whether or not metal ions were present. The amount of alkali used in each titration was 10 ml.

Exactly 50.0 ml. of a 0.005 *M* solution of divalent

\* Received August 21, 1959, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.

Abstracted from a thesis submitted by J. Pecci as a requirement for the degree of Master of Science, 1958.

This project was supported in part by a grant (A-1914) awarded by the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

† Present address: Cambridge Research Center, A. R. D. C., Bedford, Mass.



# The United States Pharmacopoeial Convention, Inc.

## ANNUAL FINANCIAL STATEMENT

Based upon the Report of the Auditor for the  
year ended December 31, 1959

### STATEMENT OF INCOME AND EXPENSE

#### Income:

Sales of Pharmacopeias:		
Collections.....	\$25,133.25	
Add—Increase in accounts receivable.....	454.90	
		\$ 23,588
Sales of reference standards:		
Collections.....	\$49,742.00	
Add—Increase in accounts receivable, and adjustment o. prior years' collections.....	2,323.15	
		52,065
Interest on investments, less amortization of bond premiums of \$22.65.....		6,839
Interest on savings deposits.....		6,012
Use of text by others.....		25
Miscellaneous.....		121
		<u>\$ 80,631</u>

#### Expense:

Revision ..	\$86,330.41	
Less—Increase in inventory.....	340.76	
		\$ 85,989
Administration.....		8,783
Publications.....	\$27,031.43	
Add—Decrease in inventory.....	10,543.02	
		37,577
Headquarters.....		\$ 5,658.97
Add—Depreciation expense		
Building.....	\$2,430.77	
Furnishings and equipment.....	4,208.00	6,639.76
		12,298
Convention.....		4,121
		<u>\$119,071</u>
Excess of Expense over Income.....		<u>\$ 38,410</u>

### STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT)

#### Receipts:

Sales of pharmacopeias.....	\$ 25,133
Sales of reference standards.....	19,712
Interest on investments.....	6,862
Interest on savings deposits.....	6,012
Use of text by others.....	25
Miscellaneous.....	121
Transfer from savings accounts.....	18,000
	<u>\$106,575</u>

	Revision	Adminis- tration	Publications	Head- quarters	Convention	Furnish- ings and Equip- ment	Total
Disbursements:							
Printing and binding.....			\$27,034.43				\$ 27,034
Salaries.....	\$57,209.72	\$5,590.20		\$1,791.20	\$ 300.20		64,891
Meetings.....	2,613.77	1,455.42			3,596.78		7,665
Supplies.....	1,113.52	46.44			240.27		1,400
Postage and telegrams.....	1,709.02	36.11			191.50		1,936
Utilities.....				2,328.71			2,328
Repairs and maintenance.....				1,351.03			1,351
General.....	23,684.38*	1,655.50					25,339
Insurance and taxes.....				185.00			185
Furnishings and equipment.....						\$178.75	178
	<u>\$86,330.41</u>	<u>\$8,783.67</u>	<u>\$27,034.43</u>	<u>\$5,658.97</u>	<u>\$1,121.75</u>	<u>\$178.75</u>	<u>\$132,707</u>
* .....		\$ 4,166					
.....		5,137					
.....		11,868					
Promotion.....		103					
Miscellaneous.....		2,410					
		<u>\$23,684</u>					

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49

JULY 1960

NUMBER 7

## The Avidity of Salicylic, Gentisic, and Salicyluric Acids for Heavy Metal Cations\*

By JOSEPH PECCI† and WILLIAM O. FOYE

Potentiometric titrations were carried out with the title compounds in the presence of various heavy metal cations. Evidence was provided by this method that  $\text{Cu}^{++}$ ,  $\text{Fe}^{+++}$ , and  $\text{Al}^{+++}$  ions were capable of forming stable chelates with these acids. No evidence of chelation was found with  $\text{Co}^{++}$ ,  $\text{Ni}^{++}$ ,  $\text{Zn}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ , or  $\text{Ag}^{+}$  ions, although chelates of low stability would not be detected by this procedure. The avidities of these acids for metal ions were recorded as stability constants, and their magnitude indicates that the chelates should be capable of existence in the animal cell.

IT HAS BEEN suggested that salicylates may exert some, if not all, of their biological effects through their ability to chelate the ions of metals (1). The nonchelating *meta*- and *para*-hydroxybenzoic acids show none of the classical actions of the salicylates (2). It was therefore thought enlightening to examine salicylic acid and its major metabolites, salicyluric and gentisic acids, in regard to the heavy metal cations that they are capable of chelating, and also to know quantitatively the avidity with which these salicylates bind metal ions.

Potentiometric titration appeared to be a suitable method for obtaining the desired information. Examination of the curves produced would indicate whether or not chelation had occurred, and stability constants could be calculated from the pH measurements. Mathematical treatment of the results was that derived by Albert (3, 4) for chelating agents having two ionizing groups. Babko (5, 6, 7) has previously obtained stability constants for salicylic acid with ferric, cupric,

and aluminum ions using a spectrophotometric procedure based on the method of Job (8).

### METHODS

**Materials.**—The metallic ions were used in the form of the following salts: Fisher certified  $\text{CuCl}_2$ , Baker analyzed reagents  $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{Ca}(\text{CH}_3\text{CO}_2)_2 \cdot \text{H}_2\text{O}$ , and  $\text{AgNO}_3$ . The *o*-hydroxybenzoic acid used was Fisher certified grade, the *p*-hydroxybenzoic acid obtained from Eastman Organic Chemicals, and the gentisic acid obtained from Matheson, Coleman and Bell. The salicyluric acid was prepared by the procedure of Hanzlik (9), m. p. 168–170°.

Carbonate-free 0.100 *N* sodium hydroxide was prepared from a filtered saturated solution of sodium hydroxide U. S. P. The stock solution was appropriately diluted with freshly boiled out and cooled distilled water, and standardized.

**Titrimetric.**—Exactly 50.0 ml. of a 0.01 *M* solution of salicylic acid or derivative was titrated with 0.100 *N* sodium hydroxide, first in the absence of metal ions and then in the presence of 0.00025 mole of a divalent metal salt or 0.000165 mole of a trivalent metal salt (one equivalent). Thus the molar concentration was 0.005 *M* for divalent ions and 0.0033 *M* for trivalent ions, and the molar ratio of salicylic acid to metal was 2:1 for divalent and 3:1 for trivalent ions. The total volume of the solution being titrated was therefore 50 ml., whether or not metal ions were present. The amount of alkali used in each titration was 10 ml.

Exactly 50.0 ml. of a 0.005 *M* solution of divalent

\* Received August 21, 1959, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.

Abstracted from a thesis submitted by J. Pecci as a requirement for the degree of Master of Science, 1958.

This project was supported in part by a grant (A-1914) awarded by the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: Cambridge Research Center, A. R. D. C., Bedford, Mass.

metal ions or 50.0 ml. of a 0.0033 *M* solution of trivalent metal ions was also titrated with 0.100 *N* sodium hydroxide. All titrations were carried out at  $20 \pm 2^\circ$ .

The solution to be titrated was contained in a beaker fitted with a stopper admitting glass and calomel electrodes, a thermometer, buret tip, and capillary tube through which nitrogen was passed continuously for stirring and maintaining an inert atmosphere.

The alkali was added in 0.5-ml. portions and the pH was recorded after each addition by means of a Beckman model H2 pH meter frequently standardized with Beckman buffer solution. Graphs were then prepared by plotting pH vs. volume of alkali.

**Expressions.**—The calculations of stability constants are based on the following expressions, since chelate formation has been shown by Bjerrum (10) to be thermodynamically reversible without appreciable energy of activation and to follow a step-wise course, and the present results, as shown by the formation curves (Fig. 1), are in agreement.

$$K' = \frac{[1:1 \text{ chelate}]}{[\text{free metal ions}] \times [\text{free chelating species}]}$$

$$K'' = \frac{[2:1 \text{ chelate}]}{[1:1 \text{ chelate}] \times [\text{free chelating species}]}$$

By combining these two equations, an overall stability constant (*Ks*) for the entire reaction is obtained (where a divalent salt is involved):

$$Ks = \frac{[2:1 \text{ chelate}]}{[\text{free metal ions}] \times [\text{free chelating species}]^2}$$

or, more conveniently,

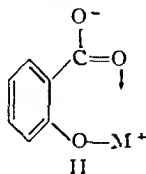
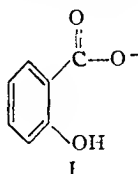
$$Ks = K'K''$$

In the case of a trivalent salt, the following expressions would, of course, be applicable:

$$Ks = \frac{[3:1 \text{ chelate}]}{[\text{free metal ions}] \times [\text{free chelating species}]^3}$$

$$Ks = K'K''K'''$$

The term "free chelating species" refers to that form of the chelating agent with which the chelate is in equilibrium. In the case of salicylic acid and a divalent metal ion the free chelating species in equilibrium with the 1:1 chelate is shown by structure I, and the free chelating species in equilibrium with the 2:1 chelate by structure II.



**Calculations.**—If we define  $\bar{n}$  as the average number of ligand molecules bound by each metal ion at any stage in the process of complex formation, and  $[Sc]$  as the corresponding concentration of free chelating species, then  $K'$  and  $K''$  can be calculated from Eqs. 1 and 2 without actual measurement of the free metal ion concentration (3, 10).

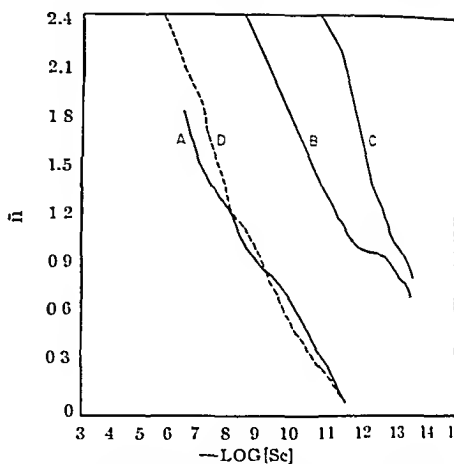


Fig. 1.—Formation curves of the salicylic acid chelates. A, Salicylic acid (0.01 *M*) and  $\text{Cu}^{++}$  (0.005 *M*); B, salicylic acid (0.01 *M*) and  $\text{Al}^{+++}$  (0.0033 *M*); C, salicylic acid (0.01 *M*) and  $\text{Fe}^{+++}$  (0.0033 *M*); D, theoretical curve for A assuming step-wise addition.

$$K' = \frac{\bar{n}}{(1 - \bar{n})[Sc]} \quad (\text{Eq. 1})$$

$$K'' = \frac{\bar{n} - 1}{(2 - \bar{n})[Sc]} \quad (\text{Eq. 2})$$

It follows then that  $K'''$  may be calculated from Eq. 3.

$$K''' = \frac{\bar{n} - 2}{(3 - \bar{n})[Sc]} \quad (\text{Eq. 3})$$

These equations can be solved because the experimental data allow the calculation of  $[Sc]$  from Eqs. 4 or 5 and  $\bar{n}$  from Eq. 6 as shown by Albert (4).

$$\log [Sc] = \log \{ [XSc^\circ] - [\text{NaOH}] - [II^{++}] \} - \log \beta \quad (\text{Eq. 4})$$

$$\log [Sc] = \log \{ 2[XSc^\circ] - [\text{NaOH}] - [II^{++}] \} - \log \beta \quad (\text{Eq. 5})$$

$$\bar{n} = \frac{[XSc^\circ] - \alpha [Sc]}{[M^\circ]} \quad (\text{Eq. 6})$$

Where  $[XSc^\circ]$  = the molar concentration of salicylate before metal ion was added (0.01 *M*);  $[\text{NaOH}]$  = the molar concentration of sodium hydroxide which would be present if salicylate and metal ion were absent;  $[H^+]$  = the hydrogen ion concentration calculated from the pH readings;  $[M^\circ]$  = the total molar concentration of metal ion, bound or free;  $\alpha = ([H^+]/K_a) + ([II^{++}]/K_a K_a') + 1$ ;  $\beta = ([H^+]/K_a) + (2[II^{++}]/K_a K_a') + 1$ ;  $K_a$  = the secondary ionization constant for the salicylate,  $K_a'$  = the primary ionization constant for the salicylate.

In order to decide whether to use Eqs. 4 or 5 to determine  $\log [Sc]$ , a comparison was made of the pH values obtained on titration with alkali in the presence and in the absence of metal. For example, in Table I, salicylic acid in the presence of  $\text{Cu}^{++}$  ions shows evidence of end point formation when two equivalents (10.0 ml.) of alkali are added because the pH obtained in the presence of  $\text{Cu}^{++}$

TABLE I.—POTENTIOMETRIC TITRATION OF SALICYLIC ACID AND CUPRIC CHLORIDE AT 20°

0.1 N NaOH, ml	pH	log [Sc]	$\bar{n}$	log K'	log K''
0	2.62				
0.5	2.68	<u>14</u> 77			
1.0	2.78	<u>14</u> 93			
1.5	2.88	<u>13</u> 09			
2.0	2.98	<u>13</u> 24			
2.5	3.12	<u>13</u> 44			
3.0	3.28	<u>13</u> 66	0.03		
3.5	3.42	<u>13</u> 84	0.01		
4.0	3.61	<u>12</u> 07	0.02		
4.5	3.88	<u>12</u> 38	0.03		
5.0	4.21	<u>12</u> 70	0.10	10.34	
5.5	4.58	<u>11</u> 07	0.25	10.45	
6.0	4.95	<u>11</u> 40	0.45	10.52	
6.5	5.32	<u>11</u> 73	0.60	10.46	
7.0	5.88	<u>10</u> 22	0.80	10.38	
7.5	6.88	<u>9</u> 14	1.00		
8.0	7.28	<u>9</u> 43	1.18		7.91
8.5	7.62	<u>9</u> 66	1.39		8.14
9.0	8.78	<u>8</u> 63	1.60		7.54
9.5	10.31	<u>7</u> 85	1.98		7.85
10.0	10.85				
log mean of antilogs				10.43	7.91

ions is only 0.9 pH units below that of the pH of salicylic acid alone. At one equivalent (5.0 ml), the pH of salicylic acid in the presence of Cu<sup>++</sup> ions is 4.27 pH units below that of salicylic acid alone.

Whenever end point formation is reached after the addition of two equivalents of alkali, Eq 5 is used. If end point formation had occurred after the addition of one equivalent of alkali, Eq 4 would have been used. In this work, all values for log [Sc] were calculated from Eq 5.

The overall stability constant was calculated from Eq 7 and then compared with an independent calculation from Eq 8.

$$\log K_s = -2 \log [Sc], \text{ where } \bar{n} = 1.00 \quad (\text{Eq 7})$$

$$\log K_s = \log K' + \lg K'' \quad (\text{Eq 8})$$

When log K'' could be calculated, log K<sub>s</sub> was calculated from Eq. 9.

$$\log K_s = \log K' + \log K'' + \log K''' \quad (\text{Eq 9})$$

### RESULTS AND DISCUSSION

Potentiometric titrations were carried out with salicylic acid in the presence of the following ions: Cu<sup>++</sup>, Fe<sup>+++</sup>, Al<sup>+++</sup>, Co<sup>++</sup>, Ni<sup>++</sup>, Zn<sup>++</sup>, Mg<sup>++</sup>, Ca<sup>++</sup>, and Ag<sup>+</sup>. Examination of the curves drawn (pH vs volume of alkali) showed that only copper, aluminum, and ferric ions were capable of chelating with salicylic acid. Gentisic and salicylic acids also were found to chelate only these three metal ions of the metals studied. The titration curves obtained with the nonchelating metals in the presence of a salicylate reproduce in turn the titration curves of the individual components, whereas the titration curve representing a chelation, where additional hydrogen ions are liberated, follows "a path independent of that of the chelating agent and is almost always independent of that of the metal ion (3)." In the latter case, an entirely new picture is presented, and furthermore, the

usual precipitates of metal hydroxides no longer take place upon the first addition of alkali. These differences in titration results from chelating and nonchelating reactions have been adequately illustrated in the past (3, 11), however, and need no elaboration here. The extent of the displacement of the curve representing a chelation forms a measure of the avidity of a particular agent for a given metal ion, and is dealt with algebraically in the section on calculations. It should be pointed out, however, that it is not easy to recognize a low value of K' by this method, so that a chelate of low stability could be overlooked.

In the titrations of the salicylates with various metal ions, pH values were obtained for each addition of alkali. From these readings, values for log [Sc] and  $\bar{n}$  were calculated from Eqs 5 or 6 and were used for the final calculation of stability constants. Table I gives a typical example of the results of a titration where chelation took place with a divalent metal ion and Table II where a chelation took place with a trivalent one. Values for log K', log K'', and log K''', calculated by Eqs 1, 2, and 3, respectively, are included, as well as values for log K<sub>s</sub> derived from both Eq 7 and Eq 8 in Table III. When log K''' could be calculated, Eqs 7 and 8 could be used to calculate log K'K'', and log K<sub>s</sub> was obtained from Eq 9.

TABLE II.—POTENTIOMETRIC TITRATION OF SALICYLIC ACID AND FERRIC CHLORIDE AT 20°

0.1 N NaOH, ml	pH	log [Sc]	$\bar{n}$	log K'	log K''	log K'''
0	2.25					
0.5	2.30	<u>15</u> 98	0.69	14.36		
1.0	2.32	<u>15</u> 99	0.82	14.67		
1.5	2.40	<u>14</u> 14	0.83	14.55		
2.0	2.45	<u>14</u> 22	0.88	14.65		
2.5	2.52	<u>14</u> 33	0.94	14.87		
3.0	2.60	<u>14</u> 46	0.99			
3.5	2.69	<u>14</u> 60	1.00			
4.0	2.75	<u>14</u> 68	1.10		12.39	
4.5	2.85	<u>14</u> 82	1.20		12.57	
5.0	2.98	<u>13</u> 02	1.21		12.41	
5.5	3.10	<u>13</u> 17	1.30		12.46	
6.0	3.20	<u>13</u> 28	1.40		12.54	
6.5	3.30	<u>13</u> 38	1.53		12.67	
7.0	3.48	<u>13</u> 58	1.60			
7.5	3.60	<u>13</u> 66	1.80			
8.0	3.80	<u>13</u> 83	1.99			
8.5	4.08	<u>12</u> 04	2.20			11.36
9.0	4.55	<u>12</u> 38	2.44			11.52
9.5	5.80	<u>11</u> 36	2.70			11.01
10.0	9.00					
log mean of antilogs				14.65	12.52	11.35

The values obtained for log K', log K'', and log K''' were converted to antilogarithms, averaged, and reconverted to logarithms. Not all values were selected for this purpose, as they are not all of equal accuracy. These results are summarized in Table III, and may be compared with literature K<sub>s</sub> values for salicylic acid chelates in Table IV.

Curves obtained by plotting values of  $\bar{n}$  against -log [Sc], as shown in Fig. 1, have been termed formation curves by Bjerrum (10), and they provide evidence as to whether a given reaction is stepwise or not. Figure 1 gives the formation curves for the chelates of salicylic acid with Cu<sup>++</sup>,



# Relationship of Chemical Structure to Central Nervous System Effects of Tranquilizing and Anticonvulsant Drugs\*

By T. C. BARNES

Isolated mice provide a method of testing tranquilizing and anticonvulsant drugs on abnormal animals. Trifluoroperazine was effective in abolishing head twitch in lower doses than chlorpromazine, showing potency of the added piperazine ring. Phenobarbital inhibited convulsive movements induced by tactile stimulation but did not abolish head twitch.

PREVIOUS REPORTS in abstract form (1, 2, 3) have described the agitated behavior of mice and rats kept in solitary confinement. For effects in another species see (4). The method could provide a bioassay for tranquilizing and anticonvulsant drugs. Keller and Umbreit (5) have reported the violent twitch of the head produced by touching the occiput in an isolated mouse. Yen, *et al.* (6), observed that a mouse after isolation will attack a normal mouse. Pavlov (7) found that some isolated dogs become neurotic, refuse food, and die. Liddell (8) stated that only the sheep and goat can withstand isolation. Lasagna and McCann (9) investigated the increased toxicity of amphetamine in agitated mice.

## EXPERIMENTAL

In the experiments reported in this paper, white mice were taken from large communities and isolated in cages 6 X 6 X 12 inches, with metal sides and mesh top. Time of isolation lasted from twenty-four hours to a year. Head twitch was present in 5% of controls but only mice without this sign of agitation were used. Time of isolation required to develop twitch varied from several days to weeks (some mice did not acquire twitch).

Unlike Pavlov's (7) isolated dogs, isolated mice did not show anorexia and a full diet of fresh meat and vegetables was provided, besides the usual rat pellets. Lee, *et al.* (10), have shown that isolated mice become agitated sooner on vitamin B<sub>12</sub>-deficient diet (the type of fat eaten is also a factor.) In our experiments anorexia occurred only with reserpine. Mice were isolated visually but could smell and hear the other mice. They were taken from stock cages containing a large number, to emphasize the factor of social isolation.

In mice, drugs were given orally with a blunt needle. Saline controls did not affect head twitch or convulsive movements. At least 40 mice were

used for each drug (mice that had recovered from a drug were tested again with a different drug). The head twitch was produced by a light touch on the occipital region (tested every one-half hour over a seven-hour period). Doses were investigated from subliminal to those killing 5% or over (dead mice counted the next day). Mice were considered sedated if they showed slow locomotion when touched, or definite ataxia, or obvious sleep. Recording devices of the Skinner box type were not possible because the mouse had to be touched lightly on trigger zone for twitch (the occiput). Records of the spontaneous movements of normal mice as influenced by drugs are of less significance than the effects of drugs on abnormal movement of "neurotic" mice.

A convulsive movement, distinct from head twitch, is defined as a violent, purposeless displacement of the body (elicited by touching the occiput).

Twenty-five tame white rats were also subjected to visual isolation in cages with metal sides (same dimensions as for mice). These were mostly old rats (104-438 Gm), taken from a large community. The head twitch is not seen in the rat but agitation is shown by vicious attack on a blunt glass rod which originally did not elicit a pugnacious attitude. Some rats squealed, or rolled over, or showed catatonic postures after isolation from several days to months.

Agitated rats are too vicious to handle during administration of drugs by stomach tube so a method of self-administration of ether was used, which we developed previously for the study of wound healing (11). An inverted glass funnel containing cotton soaked in ether in the spout, was placed over the animal standing on a sheet of fly-screen resting on a flat board. When the rat tried to escape upward, it inhaled ether and fell to the ventilated floor, thus avoiding an overdose of anesthetic. The excitement stage of etherization was avoided. Rats also ate a rich diet (except on reserpine).

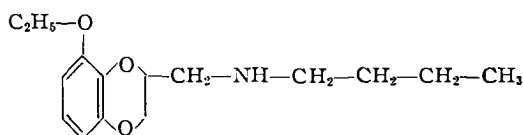
## RESULTS

Table I presents the data obtained with 19 drugs on the head twitch and convulsive movements of isolated mice. The last column gives the percentage of mice which were sedated. The potency of head twitch inhibition decreased in the following order: chlorpromazine > promazine > Lilly 26125 > reserpine > trifluoroperazine > acetazolamide > phenacemide > azacyclonol > ethotoin > meprobamate > glutamic acid lactam > primidone. The head twitch is perhaps a neurotic or psychotic sign. If so, the above compounds might be said to have tranquilizing action. On the other hand, trimethadione, methylsulimide, SKF 5627, SKF 2599, diphenylhydantoin, and phenobarbital did not inhibit the

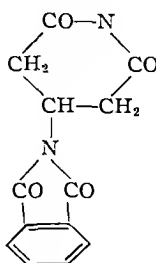
\* Received October 12, 1959, from Hahnemann Medical College, Philadelphia, Pa., and Parmac Laboratories, Dallas, Tex.

Presented to the Scientific Section, A PH A, Cincinnati meeting, August 1959.

twitch. The minus sign before the phenobarbital data indicates that head twitch was increased. Drugs having no common name that would fit the first column are as follows: Lilly 56125 is 8-ethoxy-2-butylamino-methyl-1,4-benzodioxane.



SKF 5627 is 3-phthalamido glutaramide.



SKF 2599 is reduced diphenylhydantoin. It will be seen in Table I that the so-called convulsive movement or violent displacement of the body without purpose is inhibited by an anticonvulsive drug like trimethadione, which has no effect on the head shake. Dilantin increased the convulsive movements indicated by the minus sign in the

table. The ethyl congener, ethotoin, and also the reduced forms of diphenylhydantoin did not increase convulsions. The twitch might be called a "psychomotor" phenomenon and it is of interest that phenacemide eliminated twitch in 19% of mice. However, methsuximide had no effect on twitch or convulsive movements but sedated 70% (40% of these died.) Note the dosage was carried above the 5% mortality level with this drug.

The structural change in primidone (reduced phenobarbital) eliminated exaggeration of twitch and produced less sedation. Closing the ring in glutamic acid activated this inert compound. It was surprising to find acetazolamide acting as a "tranquilizer" but the anticonvulsive action was expected. Also the anticonvulsive effect of meprobamate is in accord with its chemical use. The antitwitch action of meprobamate was weak. Azacyclonol proved to be less effective against twitch than the phenothiazines. Reserpine required the high dose of 10 mg./Kg. which produced ptosis, anorexia, and bloody diarrhea. A suspension of reserpine in water was used since reserpine injectable, Ciba, contains sufficient benzyl alcohol to affect the behavior of the mice. Thus benzyl alcohol in doses present in reserpine injectable, Ciba, at a level of 10 mg./Kg. reserpine gave the following results: antitwitch 40%, anticonvulsive 22%, sedated 7%.

Another solvent which should be avoided is propylene glycol—at dose of 3,000 mg./Kg. inhibited twitch in 20% and sedation in 30% (no convulsive mice were available at the time.)

As a control for the phenothiazines, promethazine was tested at 25 mg./Kg. with no effect. To compare with the effects of acetazolamide, chlorothiazide at 800 mg./Kg. was found to have no effect on behavior. As a control for Lilly 26125, phenoxybenzamine 1 mg./Kg. was without effect on twitch or convulsive movement. Statistical analysis by Dr. J. C. Munch on 16 drugs given in Table I (with the exception of promazine and trifluoperazine) shows that the coefficient of correlation  $r$  for antitwitch to anticonvulsant is 0.52, antitwitch to sedation 0.06, and anticonvulsant to sedation is 0.22.

Only 25 isolated white rats were studied so the results will be presented very briefly. Shortest time for developing aggressive behavior was twenty-nine hours, others remained tame for many months. Reserpine, oral suspension, was given at 1 to 5 mg./Kg. doses and prevented the attack on the glass rod in 50% of the agitated animals. In some, even after a total of 100 mg./Kg. reserpine over several days, the neurotic behavior persisted. Ptosis, hyena posture, and diarrhea resulted.

## DISCUSSION

In experimental pharmacology, isolation in mice produces a head twitch which is inhibited by tranquilizing drugs. The effect of the added piperizino ring is seen in the lower dose of trifluoperazine compared with chlorpromazine. It is possible that a larger ring structure covers more receptors, protecting them from the stimulating action of neurohumoral agents. The 3 carbon and nitrogen sidechain at position 10 in the phenothiazines may attach to the same receptors that are used by the same structure in norepinephrine (12.) Once attached,

TABLE I.—EFFECT OF DRUGS ON AGITATED BEHAVIOR OF ISOLATED MICE<sup>a</sup>

Drug <sup>b</sup>	Oral Dose, mg./Kg.	Head Twitch Inhibited, %	Anti-convulsant Movement or Convulsion Stopped, %	Sedation, %
Chlorpromazine	3	70	90	20
Promazine	50	58	78	80
Lilly 26125 <sup>c</sup>	12	50	87	60
Reserpine	10	45	47	54
Trifluoperazine	1	38	53	0
Acetazolamide	800	37	50	33
Phenacemide	1500	19	47	33
Azacyclonol	250	17	20	18
Ethotoin	400	15	33	77
Meprobamate	200	11	42	50
Glutamic acid lactam	800	11	58	24
Primidone	200	6	30	56
Trimethadione	800	0	53	27
Methsuximide	500	0	0	70
SKF 5627 <sup>c</sup>	400	0	0	59
SKF 2599 <sup>c</sup>	200	0	0	25
Diphenylhydantoin	200	0	-55	75
Phenobarbital	200	-14	60	85

<sup>a</sup> All experiments include 40 or more mice. Doses are optimum for inhibition of head twitch, data based on time for peak effect, 7-hour test intervals. If mouse reacted to tactile stimulus by violent displacement of body, movement was classed as convulsive.

<sup>b</sup> Drugs are listed in decreasing effect on head twitch; most effective action is that which inhibits the greatest percentage of mice with head twitch without producing more than 5% deaths.

<sup>c</sup> See text for chemical structure.

the inert rings would protect adjacent receptors. The antitwitch action of Lilly 26125 with benzo-dioxane structure suggests that an epinephrine-like compound may be involved in the agitated mice. However, this is not a simple matter of direct adrenolytic action since phenoxybenzamine is inactive. Possibly, drug must block adrenergic receptors in the nervous system, not in the vascular system. Depletion of norepinephrine in nerves by reserpine (13) is in accord with this hypothesis. However, the persistence of some aggression in rats after a total of 100 mg./Kg. of reserpine over several weeks, suggests that there are other factors besides chemical.

Minor technical points are the ptosis with reserpine which may aggravate the sensory deprivation or isolation stress and the chirping which occurs with 100 mg./Kg. promazine which may act in the opposite direction.

The diphenyl methane structure appears weak in agitated mice as judged by the effect of azaeyclonol.

### SUMMARY

1. Isolated mice provide a method of testing tranquilizing and anticonvulsant drugs.

2. Trifluoperazine was effective in abolishing head twitch in lower doses than chlorpromazine, showing potency of added piperazine ring.

3. Phenobarbital did not abolish head twitch in mice as did the tranquilizers. The reduced form had some antitwitch action.

4. The well-known exciting effect of diphenylhydantoin in mice is abolished by reduction or by ethyl congener.

5. Closing the structure of glutamic acid endows antitwitch and anticonvulsive properties.

6. Statistical analysis shows antitwitch effect distinct from anticonvulsive effect.

### REFERENCES

- (1) Barnes, T C, and Munch, J C, *Anat Record*, 132, 409(1958)
- (2) Barnes, T C, *Federation Proc.*, 17, 347(1958)
- (3) Barnes, T C, *ibid*, 18, 368(1959)
- (4) Barnes, T C, *J Gen Physiol*, 25, 249(1941)
- (5) Keller, D L, and Umbreit, W W, *Science*, 124, 723(1956)
- (6) Yen, H C Y., Stranger, R L, and Millman, N, *J Pharmacol Exptl Therap.*, 122, 85(1958)
- (7) Pavlov, I P, "Conditioned Reflexes," Oxford University Press, London, England, 1928
- (8) Liddell, H S, "Emotional Hazards in Animals and Man," C C Thomas Publishing Co, Springfield, Ill, 1956, p 380
- (9) Lasagna, L, and McCann, W P, *Science*, 125, 1241(1957).
- (10) Lee, Y C P, Jardetzky, O, King, J T, and Vischer, M. B, *Proc Soc Exptl Biol Med*, 95, 204(1957)
- (11) Barnes, T C, and Amoroso, M, *Am. J Surg*, 87, 805(1954)
- (12) Himwich, H E, *Am J Psychiat*, 115, 756(1959)
- (13) Muscholl, E, *Klin Wochschr*, 37, 217(1959)

## Complexing Tendencies of Saccharin in Aqueous Solutions\*

By J. R. MARVEL† and A. P. LEMBERGER

Contrary to what might be expected, relatively weak complexing tendencies were observed between saccharin and various substances in aqueous solutions. A 1:1 complex is formed between saccharin and theophylline. Saccharin forms water-soluble complexes with caffeine, various amides, and phenols. No interactions were observed between saccharin and N-methylpyrrolidone,  $\gamma$ -butyrolactone and polyethylene glycol 4000.

IN RECENT STUDIES by Higuchi and co-workers (1-4) it has been shown that a variety of acidic compounds form molecular complexes with certain substances, such as caffeine, N,N,N',N'-tetramethylphthalamide, polyvinylpyrrolidone, and polyethylene glycol 4000. Saccharin, which has been widely used as a synthetic sweetening

agent for over a half century, possesses acidic properties and might be expected to show similar complexing behavior. However, Rogenbogen's report (5), in 1918, of an association between saccharin and antipyrine is the only literature citation of an organic complex formation involving saccharin.

Higuchi and Lach (4) have proposed that molecular interactions similar to caffeine complexes may be the result of an association between an acidic hydrogen and the electronegative center of the complexing agent. They also proposed that complexing may be enhanced by the high internal pressure of water which predisposes the interacting molecules to a stronger association. On the basis of this theory of complexing saccharin might be expected to show an attraction for compounds having a good electron donating center, such as the carbonyl group of an amide. It was the purpose of this study to investigate

\* Received August 21, 1959, from the University of Wisconsin, School of Pharmacy, Madison

† Fellow of the American Foundation for Pharmaceutical Education. Present address: McNeil Laboratories, Inc., Philadelphia, Pa

Presented to the Scientific Section, A Ph. A, Cincinnati meeting, August 1959.



possible interactions between saccharin and various typical complexing agents.

The solubility method (1) was employed as the analytical procedure for the observation of the complexing tendency of saccharin with the investigated complexing agents. The solubility method was reported to be particularly applicable for detecting small complexing tendencies.

## RESULTS AND DISCUSSION

**Interactions With Amides.**—All the investigated amides are freely soluble in water and except for *N,N*-dimethylacetamide, a high boiling liquid, are white crystalline solids. The observed solubility of saccharin in water at 30° with various concentrations of acetamide, *N,N*-dimethylacetamide, *N,N*-dimethylbenzamide, *N,N,N',N'*-tetramethylfumaramide, and *N,N,N',N'*-tetramethylphthalamide is shown in Fig. 1. The amount of saccharin used in these studies was 0.5 Gm. per 50 ml. of water.

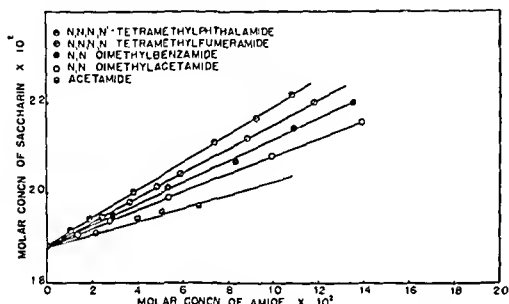


Fig. 1.—Solubility of saccharin in water at 30° as a function of amide concentration.

It was evident that saccharin associated with these amides to form soluble complexes. The linear relationship between the molar concentrations of the amide and the molar solubility of saccharin indicated a first-order dependency between the amide and the complex. Since all the amide complexes were found to be water soluble, it was impossible to calculate their actual stoichiometries. To facilitate a comparison of the complexing tendencies of saccharin with the amides, 1:1 stoichiometric relationships were assumed and overall stability constants for the complexes were calculated (6).

Since the ionization constant for saccharin is  $2.5 \times 10^{-2}$ , a considerable concentration of saccharate ions will be present in an aqueous solution of saccharin. It is possible that either undissociated saccharin or saccharate ion could associate with the complexing agents employed; therefore, the stability constants listed throughout this paper must be considered as overall stability constants.

The stability constants listed in Table I are considerably lower than the constants reported by Kostenbauder and Higuchi (2) in an investigation of *N,N,N',N'*-tetramethylphthalamide and *N,N,N',N'*-tetramethylfumaramide with *p*-hydroxybenzoic acid and salicylic acid. It is evident that the extent of complex formation in the saccharin-amide systems is relatively small.

TABLE I.—OVERALL STABILITY CONSTANTS FOR INTERACTIONS OF SACCHARIN WITH CERTAIN AMIDES IN WATER

Amide	Stability Constant
<i>N,N,N',N'</i> -Tetramethylphthalamide	1.6
<i>N,N,N',N'</i> -Tetramethylfumaramide	1.4
<i>N,N</i> -Dimethylbenzamide	1.3
<i>N,N</i> -Dimethylacetamide	1.2
Acetamide	0.6

It is of interest to note that although only small differences in the stability constants are observed, the order in which they increase is compatible from a qualitative standpoint with theoretical expectations. The unsubstituted amide, acetamide, has been shown to form hydrogen bonds (7). If, as one might expect, such associations occur in aqueous solutions, they may well be in direct competition with the association between saccharin and amide. Alkyl substitution for amide hydrogen atoms precludes the possibility of formation of such hydrogen bonds. In addition, alkyl groups have a greater tendency to release electrons by the inductive effect than hydrogen atoms. With alkyl substitution the electronegativity of the carbonyl oxygen should be intensified and the ability of the amide to complex with an acidic substance should increase. As the complexing agents are rendered more hydrophobic, the "squeezing out" effect exerted by water molecules on a complex should be enhanced (4).

**Interactions With Xanthines.**—The effects of caffeine, theophylline, and theobromine on the solubility behavior of saccharin in water at 30° are shown in Fig. 2. The quantity of saccharin employed in these studies was 0.5 Gm. per 50 ml. of water.

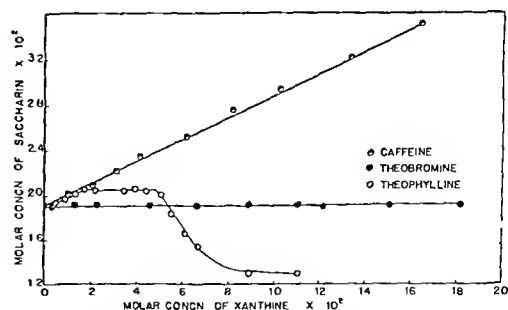


Fig. 2.—Solubility of saccharin in water at 30° as a function of xanthine concentration.

From the phase diagrams it is seen that caffeine and theophylline entered into definite complex formations with saccharin while theobromine apparently did not complex. Stability constants calculated from the initial linear portions of the phase diagrams gave values of 5.7 and 5.6 for theophylline and caffeine, respectively, when 1:1 interactions were assumed. Since the stability constants for the two xanthines are identical within experimental error, it appears that the caffeine and



# An Investigation of Solvent Effect Upon Saccharin Complexes With N,N-Dimethylacetamide<sup>\*</sup>

By J. R. MARVEL<sup>†</sup> and A. P. LEMBERGER

Complex formation between saccharin and N,N-dimethylacetamide appears to have a first-order dependency upon amide concentration in water, chloroform, and benzene. Relatively strong complexing occurs in benzene. In carbon tetrachloride and carbon tetrachloride-ethyl alcohol mixtures it appears that higher order complexes are formed. Increasing concentrations of ethyl alcohol decrease complexing tendencies through competition with N,N-dimethylacetamide for saccharin in solution.

IN A PREVIOUS communication (1), it was shown that saccharin formed weak complexes in aqueous media with certain amides, such as N,N-dimethylacetamide and N,N,N',N'-tetramethylphthalimide. Complexing of this nature was proposed by Higuchi and Lach (2) to be the result of dipolar interactions supplemented by the "squeezing out" effect by water molecules. Since saccharin possesses acidic hydrogen and these amides have good electron-donating centers, the results were somewhat unexpected.

Water is a strong dipole; thus, in an aqueous solution saccharin, its dissociation products, and an amide would probably be highly associated with water molecules. It was felt that in certain nonaqueous solvents where competing solute-solvent interactions would not be as significant the dipole-dipole interaction of saccharin and an amide might be rendered more favorable.

The present investigation is an observation of the complexing tendency of saccharin with N,N-dimethylacetamide in water, chloroform, benzene, carbon tetrachloride, and carbon tetrachloride-ethyl alcohol mixtures. The solubility method (3) was employed as the procedure for the detection of complex formation.

N,N-Dimethylacetamide was selected as the amide complexing agent because it exists only as a monomeric unit and possesses suitable solubility in the investigated solvents. This amide forms very weak complexes with saccharin in water; therefore, any increase in complexing tendency observed between this amide and saccharin in the nonaqueous solvents relative to water would be readily apparent.

## RESULTS AND DISCUSSION

**Interactions in Benzene, Chloroform, and Water.**—The effect of various concentrations of N,N-dimethylacetamide on the solubility of saccharin at 30° in benzene, chloroform, and water is shown in Fig. 1. The solubilities at 30° of saccharin in benzene, chloroform, and water are  $4.3 \times 10^{-3}$ ,  $2.04 \times 10^{-2}$ , and  $1.90 \times 10^{-2}$  moles per liter, respectively.

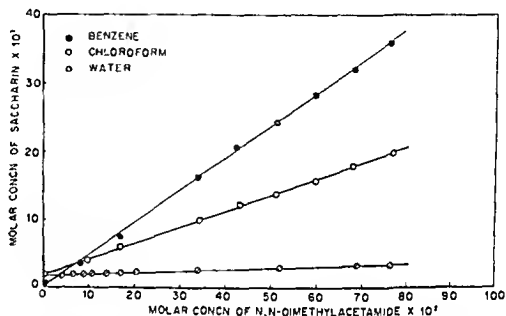


Fig. 1.—Solubility of saccharin in benzene, chloroform, and water at 30° in the presence of N,N-dimethylacetamide.

Since only soluble complexes are obtained in these solvents, the stoichiometry of the observed complexes can not be determined. However, the linear relationship which exists between the molar concentration of saccharin and the molar concentration of N,N-dimethylacetamide in each system appears to indicate that the principal complex has a first-order dependency with respect to the amide concentration. These linear relationships, although common to the three systems, do not necessarily indicate that the same complex is formed in all three solvents.

It is apparent that a quantitative comparison of the effects of these solvents upon the saccharin-amide complex would be difficult. Qualitatively the systems may be compared by determining the solubility of saccharin above its initial solubility for each system at a given amide concentration. Taking the increase in water solubility of saccharin by the amide as unity, a ratio of 31:16:1 is found for benzene, chloroform, and water, respectively. It is interesting to note that this ratio closely parallels the decrease in the polarity of the solvents. The ratios of the total molar concentration of saccharin to its initial molar solubility at 0.6 *M* N,N-dimethylacetamide are 66, 8, 1.5 for benzene, chloroform, and water, respectively. That is, the total saccharin in solution in the presence of 0.6 *M* amide in benzene is 66 times the solubility of saccharin in pure benzene. It would appear, then, that competitive interactions in aqueous systems inhibit saccharin-amide complex formation. Further, a

<sup>\*</sup> Received August 21, 1959, from the University of Wisconsin, School of Pharmacy, Madison.

<sup>†</sup> Fellow of the American Foundation for Pharmaceutical Education. Present address: McNeil Laboratories, Inc., Philadelphia, Pa.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

comparison of the results in benzene and chloroform indicates that in chloroform, solute-solvent interactions occur to reduce complex formation.

**Interaction in Carbon Tetrachloride and Ethyl Alcohol-Carbon Tetrachloride Mixtures.**—The solubility of saccharin in these solvents at 30° in the presence of varying concentrations of *N,N*-dimethylacetamide is illustrated in Fig. 2. The initial solubilities of saccharin are  $0.82 \times 10^{-2}$ ,  $1.68 \times 10^{-2}$ ,  $3.16 \times 10^{-2}$ , and  $6.47 \times 10^{-2}$  moles per liter in carbon tetrachloride, 5, 10, and 20% absolute ethyl alcohol by volume, in carbon tetrachloride, respectively.

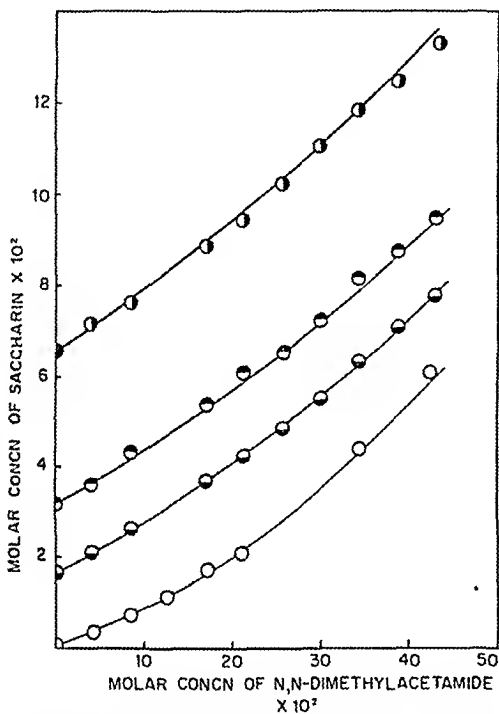
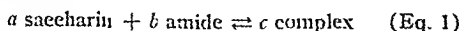


Fig. 2.—Solubility of saccharin in carbon tetrachloride-absolute ethyl alcohol v/v mixtures at 30° in the presence of *N,N*-dimethylacetamide. Curves are theoretical and points are experimental values. O, 0% ethanol; ◐, 5% ethanol; ●, 10% ethanol; and ⊙, 20% ethanol.

The relationships in Fig. 2 between the molar solubility of saccharin and the molar concentration of amide indicate that significant amounts of more than one complex form in each system. Further, the relative amount of each varies depending upon which amide concentration is selected.

The interaction between saccharin and the amide complexing agent to form a complex may be expressed as



The stability constant for this reaction is then

$$K = \frac{(\text{complex})^c}{(\text{saccharin})^a (\text{amide})^b} \quad (\text{Eq. 2})$$

The total concentration of saccharin in solution,  $S_t$ , at any molarity of amide will be equal to the sum of the concentration of free saccharin, which is

the solubility of saccharin,  $S_o$ , in the pure solvent, and the concentration of complexed saccharin. If it is assumed that two principal complexes are present in each system of Fig. 2, with the initial complex having a 1:1 and the second complex a 1:2 molecular ratio with respect to the saccharin and the amide, the total concentration of saccharin, as a function of free amide concentration,  $C$ , can be expressed mathematically as

$$S_t = S_o + K_1 S_o C + K_2 S_o C^2 \quad (\text{Eq. 3})$$

or

$$S_t - S_o = K' C + K'' C^2 \quad (\text{Eq. 4})$$

where  $K' = K_1 S_o$  and  $K'' = K_2 S_o$ .

Since complexing is weak in these systems, it may be assumed that the total concentration of the amide,  $C_t$ , is approximately equal to the concentration of the free amide.

Equation 4 can be rewritten in the following form

$$S_t - S_o = K' C_t + K'' C_t^2 \quad (\text{Eq. 5})$$

The values for the constants  $K'$  and  $K''$  were obtained by making a plot of  $S_t - S_o/C_t$  against  $C_t$ . This plot gave a straight line with an intercept of  $K'$  and a slope of  $K''$ . The values obtained are tabulated in Table I and they were used to calculate the theoretical lines of Fig. 2. The points in Fig. 2 represent experimental values. It is recognized that in these solvents saccharin may be present as a dimer; however, the order in which the apparent values for  $K_1$  and  $K_2$  increase and their relative magnitudes should not be affected since the thermodynamic activity of saccharin is a constant.

TABLE I.—APPARENT STABILITY CONSTANT FOR ASSUMED 1:1 AND 1:2 COMPLEXES IN CARBON TETRACHLORIDE-ALCOHOL SOLVENT SYSTEMS

Ethyl Alcohol Content, % by Volume	$K_1$	$K'$	$K_2$	$K''$
20	2.0	0.13	1.2	0.08
10	3.5	0.11	2.5	0.08
5	6.0	0.10	6.0	0.10
0	73	0.06	220	0.18

The intent of the above calculations was not to obtain absolute values for  $K_1$  and  $K_2$  but rather to ascertain relative values by which the systems could be qualitatively compared. From these values, it would appear that the apparent complexing tendency between saccharin and *N,N*-dimethylacetamide increases as the competition from a component in the solvent decreases. As one might suspect in a competitive system, the effect of alcohol on the saccharin-amide complex appeared to be additive in the concentration range investigated. It would also appear that the alcohol content had a greater influence on  $K_2$  than  $K_1$ .

## EXPERIMENTAL

**Reagents.**—*N,N*-Dimethylacetamide, Eastman Kodak "white label" (4972); carbon tetrachloride, reagent grade; benzene, thiophene free; ethanol,

absolute, analytical grade; and sacharin U. S. P., recrystallized from acetone, m. p. 226–228°.

**Procedure.**—Excess quantities of saccharin were placed in 10-cc. Kimble clear-glass ampuls. Varying volumes of a standard amide solution and sufficient solvent to make 10 cc. were added. With benzene, prior to sealing, the ampuls were placed in a mixture of dry ice and acetone to solidify the contents. This procedure prevented loss of the solvent and reduced the fire hazard during the sealing operation. The sealed ampuls were agitated for

forty-eight hours in a constant temperature bath at 30°. After equilibration, aliquot portions of the supernatant liquids were removed and diluted with 95% alcohol for spectrophotometric analysis at 285 m $\mu$ .

## REFERENCES

- (1) Lemberger, A. P., and Marvel, J. R., *THIS JOURNAL*, 49, 417(1960).
- (2) Higuchi, T., and Lach, J. L., *ibid.*, 43, 465(1954).
- (3) Higuchi, T., and Lach, J. L., *ibid.*, 43, 527(1954).

# The Preparation and Use of Radioiodinated Congo Red in Detecting Amyloidosis\*

By MANUEL TUBIS, WILLIAM H. BLAHD, and ROBERT A. NORDYKE

Congo red concentrates in amyloid tissue. This study was undertaken to prepare a radioactive congo red which would concentrate in amyloid, permitting external counting and, thereby, scanning and localization, as well as blood concentration measurement. Two methods were used to trace-label the dye to an adequate specific activity for such studies. Injection into guinea pigs with amyloidosis resulting from induced chronic scurvy indicated deposits and radioactivity in the liver and other organs. The "tagged" dye shows promise for locating amyloid and may serve as a means for evaluating drugs for the resorption of amyloid. The method of direct radioiodination is applicable to other similar chemical compounds.

THE OBJECT of this study was to trace-iodinate Congo red so that the accumulation of the dye in amyloid tissue would permit external counting and blood disappearance measurements.

External counting would also permit the delineation of the deposits and provide a means for evaluating drugs which might cause a massive resorption of the deposits.

Amyloid is a carbohydrate, lipid, and protein complex which is deposited in vital organs following prolonged, infective, and tissue-destructive processes and may affect the structure and functions of these organs. Most typical amyloid is stained by Congo red and its fixation and retention is the basis for a presently used clinical test for amyloidosis. The test measures the unabsorbed circulating dye and requires several venipunctures and colorimetric determination of plasma concentration (1). These procedures would be eliminated by a satisfactory method of measuring blood concentration by external counting over a representative vascular bed, such as the side of the head.

Two methods of labeling the dye were devised. The first was based on the method of Martin and Bang (2), who prepared tetraiodinated Congo red as a radiographic opaque medium using stable iodine. The second method was that of direct iodination of the dye using  $I^{131}$  and  $I^{127}$ .

A suitable test animal for *in vivo* localization was required and it had been reported (3, 4) that guinea pigs maintained on subminimal daily amounts of vitamin C low enough to produce chronic scurvy, develop amyloidosis. The distribution of congo red in such animals as well as in normal guinea pigs was studied.

## EXPERIMENTAL

Synthesis I of  $I^{131}$ -labeled Congo red was based on that of Martin and Bang (2). Benzidine was trace-iodinated using sodium radioiodide, potassium iodide, and iodine in acid solution. The iodinated benzidines were then diazotized and coupled with sodium naphthionate to give a trace-iodinated Congo red.

In a typical preparation, 384 mg. benzidine hydrochloride yielded 291 mg. of mixed, trace-iodinated benzidines with a specific activity of 2.59  $\mu$ c./mg., representing a 4.5% utilization of the original 20 mc.  $I^{131}$  used, corrected for two days decay during preparation. The product was a purplish colored crystalline mass which did not

\* Received August 21, 1959, from the Radioisotope Service, Veterans Administration Center, Los Angeles, and the Departments of Radiology and Medicine, University of California Medical Center, Los Angeles, Calif.

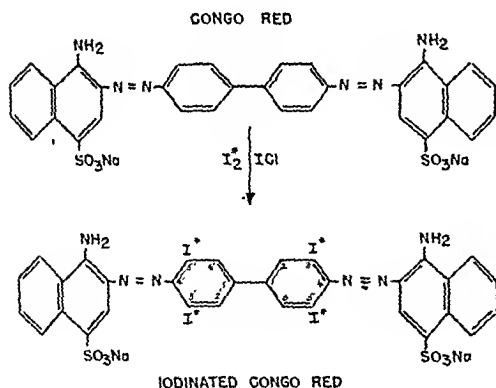
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

melt at 300°; insoluble in water, carbon tetrachloride, or acetic acid but soluble in methanol and ethanol.

The labeled Congo red was prepared by diazotizing the radioiodinated benzidines and coupling with sodium naphthionate. The dye was extracted and when finally purified had a specific activity of 0.22  $\mu\text{c.}/\text{mg.}$  The iodine content by the Kingsley and Schaffert micromethod (5) was 17.5% whereas the iodine content of a tetraiodinated Congo red would have been 42.3%, as reported by Martin and Bang (2). This indicated that our Congo red had, on the average, only 1 atom I per molecule. The yield of finally purified dye was only 34 mg. or 8.9% of the iodinated benzidines used and the activity represented only 0.4% of the original  $\text{I}^{131}$  activity used, corrected for decay.

**Synthesis II of  $\text{I}^{131}$ -Labeled Congo Red.**—In order to obviate the necessary shielding and manipulation as well as the time-consuming procedures which resulted in low yields and loss of activity of radioiodine, a second synthesis was devised. Inasmuch as the same positions in the benzidine moiety of the dye were assumed to be available for iodination, "direct iodination," using  $\text{I}^{131}$ , of the pure dye was tried, and found to yield a pure product of high specific activity, capable of rapid preparation and purification. In this method, Congo red U. S. P. in alcoholic suspension was iodinated with a chloroform solution of  $\text{I}^{131}$  of high specific activity in the presence of iodine monochloride and the final dye washed with appropriate solvents, after which adsorbed iodine was removed by dialysis or anion exchange. This reaction is shown in the following equation:

### SYNTHESIS II



The radioiodine is shown to be in the 3, 3', 5, and 5' positions but since it is trace-iodinated, it may be in any one or more of these positions in any of the molecules.

A typical preparation was as follows: 85 mg. of Congo red U. S. P. was suspended in 30 cc. absolute ethanol, in a 100-cc. round-bottom flask connected by a ground-glass joint with a reflux condenser. In a 25-cc. Squibb-type separator were placed 2 cc. of water-washed chloroform which had been dried over anhydrous sodium sulfate. To the funnel were then added 0.33 cc. of 0.01 *M* potassium iodide solution, 0.20 cc. of 2.5 *M* hydrochloric acid, and 0.10 cc. of *M* sodium nitrite and

approximately 40 mc.  $\text{I}^{131}$  solution in a volume of 1 to 3 cc. The funnel was swirled to mix the aqueous layer, then shaken vigorously to extract the liberated  $\text{I}^{131}$  and  $\text{I}^{127}$  by the chloroform. The chloroform solution was drained carefully into another similar funnel, avoiding any of the aqueous supernate, and the addition of similar quantities of the reagents except the  $\text{I}^{131}$  was repeated. The funnel again was shaken and the second chloroform extract combined with the first, and washed with two or more 2-cc. portions of water until the pH was not less than 5 to 5.5 (test paper). The chloroform solution was added to the flask containing the Congo red and to this was added 0.2 cc. of a chloroform solution containing 0.0163 mg. of iodine monochloride. The condenser was attached and the contents actively refluxed in a water bath for eight to twelve hours. At the end of this time, the condenser was removed and the solvents evaporated nearly to dryness in a well ventilated hood. The pasty residue was loosened and transferred to a small M-porosity sintered-glass filter with the aid of 20 cc. of water-washed, dried chloroform and further washed with 20 cc. of a mixture of three volumes of ethyl ether and one volume absolute ethanol, saving all washes for decay and safe disposal. The dye was air dried. The recovery was practically quantitative.

The dye contained small quantities of adsorbed  $\text{I}^{131}$  which were removed by dialysis vs. multiple 4-L. changes of distilled water adjusted to pH 7.2 to 7.6 and containing about 1 mg. sodium iodide. Dialysis was continued until a 5-cc. portion of the dialysate showed a count of one to two times that of background, usually requiring 10 or so dialysis periods of one hour each. The per cent dialyzable  $\text{I}^{131}$  of the final dye was ca. 0.5% of the total activity.

An alternate, preferable procedure for the removal of adsorbed  $\text{I}^{131}$  was the use of a column of anion exchange resin Amberlite IRA-400<sup>1</sup> in either the OH or Cl form, analytical grade, 30-50 mesh. The resin was prepared by first washing until the wash water was color free, then completely saturating with dye by soaking in 1% Congo red U. S. P. for several days. The column was made of 4-mm. glass tubing i. d., 35 cm. long, and contained ca. 1 Gm. of the dried, prepared resin. The column was washed with 1% Congo red solution just prior to use and then the labeled dye in 1% concentration passed through. No abstraction of labeled dye occurred. A single passage removed unbound  $\text{I}^{131}$  iodide as evidenced by later dialysis, ascending paper chromatography in 70% alcohol, and radioautography. The pH of the effluent labeled dye was 9.5 after use of the IRA-400 (OH) form and 7.1 to 7.5 after IRA-400 (Cl) form and the pH was adjusted to meet U. S. P. XV requirements of Congo red injection, i. e., 7.0 to 9.0. The dye solution was sealed in serum vials and sterilized in boiling water for thirty minutes.

The acute intravenous toxicity  $\text{LD}_{50}$  was 3.8 mg./20 Gm. mouse, as determined by an independent laboratory in a manner similar to that of Somers and Whittet (6).

By the direct iodination method, in one run, the

<sup>1</sup> Product of Rohm and Haas Co., Philadelphia 5, Pa. Available from Fisher Scientific Co., Pittsburgh, Pa.

specific activity of the final purified dye was 22.1  $\mu\text{c./mg.}$  and the iodine content was 0.52% (5), indicating a ratio of 1 atom iodine per 35 molecules of dye. In this run, the final weight of unpurified dye was 91.4 mg. obtained from 85 mg. Congo red originally used. This represented a utilization of 5.2% of the  $\text{I}^{131}$  used, when corrected for decay. The average specific activity of the resin-purified material was 9.7  $\mu\text{c./mg.}$  The purified dye was found to have the identical absorption maximum, i. e., at 500  $\text{m}\mu$ , solubilities and chromatographic behavior as the unlabeled dye.

**Studies of Distribution in Scorbatic and Normal Guinea Pigs.**<sup>2</sup>—Male guinea pigs weighing from 200 to 400 Gm. were divided into groups of normals and those to be fed a chronic scorbutogenic diet. The normals were fed *ad libitum* an adequate diet consisting of Rockland guinea pig diet ("C" Fortified)<sup>3</sup> which contained "vitamin C fortified to guarantee 8 mg. daily" per pound. In addition, these controls were given adequate daily supplements of vitamin C orally, approximately 6 mg. The daily weight gain was 4 to 6 Gm. and they thrived.

The "scurvy" guinea pigs were fed *ad libitum* a scorbutogenic diet of Rockland rabbit ration<sup>3</sup> which contained "vitamin C, inherent trace." In addition, they were given vitamin C orally, 0.1 to 0.2 mg. in one series and 0.4 to 1.0 mg. in a later series, to maintain the animals in the chronic stage of the disease.

When the vitamin C-deficient guinea pigs showed one or more of the symptoms of scurvy, they were maintained for periods of one to three weeks so as to prolong the disease and allow the development of amyloidosis. The average number of days on the scorbutogenic diet was twenty-four in one series and thirty-eight in a later series.

When the animals were considered suitable for the test, the normal and chronically scurvy animals were weighed and injected intraperitoneally with Nembutal<sup>4</sup> at the dosage of 12 mg./454 Gm. followed by the labeled Congo red at the dosage of 1 mg./454 Gm. body weight, the human dosage suggested by Unger, *et al.* (1). The doses administered varied from 0.43 to 1.29 mg., in volumes of 0.11 to 0.42 cc., and represented 0.96 to 8.82  $\mu\text{c.}$  These were injected into a femoral or visceral vein after exposure. Later, it was found more convenient to inject via a lateral vein in the penis. This prevented contamination of the viscera and counting of this organ showed that very little of the dose remained at the site of injection. Serial blood samples were taken from the heart or hepatic veins at early times of the order of two to five minutes after injection, and thereafter at intervals such as ten, twenty, and thirty minutes, and at sacrifice, which was thirty to forty minutes for the first series and forty to fifty-five minutes for the second series. The guinea pigs were killed by exsanguination, and at post-mortem the organs were removed and weighed. Their gross appearance was noted and sections were taken for microscopic studies as well as for weighing in capped counting tubes for

radioactivity estimations in a well-type scintillation counter.<sup>5</sup> The radioactivities of the organs and fluids were calculated on the basis of counts/minute/Gm. or counts/minute/cc. From these values were calculated the per cent of injected dose for normal and scorbutic guinea pigs in Table I.

A summary of the qualitative difference between normal and scorbutic animals would be as follows: the distribution of labeled Congo red is generally in the same range with considerable overlap except for liver, lung, and thyroid which seems to be about twice as high in the scorbutic.

In some of the scorbutic animals, the livers were perfused and retained their radioactivity despite the removal of the blood. Grossly, sections were visibly stained by the dye which was more visible in long-time frozen slices. The spleen and adrenals of the scorbutic animals were usually enlarged and abnormal in texture but had only as much activity per Gm. as the normals. The liver of the scorbutic animal had a greater activity than the normal, but the kidney activity was of the same order. According to Anderson (7) the adrenals and spleen, as well as liver and kidney, are the organs most frequently the site of deposition of amyloid. Richardson (8) using very much larger doses of unlabeled Congo red in rabbits and cats indicated that the dye in "the liver, spleen, lungs, kidneys, skin, and so on, is partially bound, physically or chemically, by some cellular or noncellular constituent, otherwise its concentration would remain in equilibrium with that of the blood." This is confirmed by our data.

A comparison of the clearance of our labeled dye from the blood of normal and scorbutic animals shows some variance with that reported in the literature. Both our normal and scorbutic animals showed about 80% removal from the blood between thirty-five to fifty-five minutes. This is in general agreement with Knorpp, *et al.* (9), who prepared Congo red labeled with  $\text{S}^{35}$  and used this in human subjects and reported 85% removal in ten to thirty minutes, indicating that injected small amounts are removed rapidly. Richardson (8) showed that rabbits injected intravenously with 50 mg./Kg. cleared approximately 25% from the blood in the first hour, despite this high dosage level. This agrees with Bennhold (10) who reported a 20% removal in man in the first hour, with a dosage level of 100 mg. Unger, *et al.* (1), using a dosage level of 1 mg. per pound of body weight in normal human subjects, reported 14% removal in the first thirty minutes, and 76% removal in the case of amyloid patients.

The variance of our results from those of Richardson (8) may be explained, aside from a species difference, by the dosage levels, wherein he administered 23 mg. per pound of body weight to rabbits and 204 mg. per pound to cats. Our dosage level of 1 mg. per pound, the common human dosage, might be considered "physiological" and did not cause "overloading" of the excretory systems.

<sup>2</sup> The authors wish to acknowledge gratefully the expert technical assistance of Mr. Edward Posnick and Mr. Laurence V. Johnson in these studies.

<sup>3</sup> Distributed by A. E. Staley Mfg. Co., Decatur, Ill.

<sup>4</sup> Abbott Laboratories, North Chicago, Ill.

<sup>5</sup> Tubes used were screw-cap culture tubes 16  $\times$  125 mm. The crystal used was 2  $\times$  2 inches, sodium iodide, thallium activated. The scaler was SC-51 Autoscaler, Tracerlab, Boston, Mass., and had four SC-41T binary plug-in units. The input circuit had a 1 microsecond resolving time. In a 5-cc volume, samples had ca. 700,000 counts/minute/microcurie of  $\text{I}^{131}$ .

TABLE I.—PER CENT INJECTED DOSE OF CONGO RED- $I^{131}$  PER GM. OF TISSUE OR CC. OF FLUID

Tissue	Normal		Scorbutic	
	Average	Range	Average	Range
Blood 2'-5' <sup>a</sup>	1 65 (3) <sup>b</sup>	1.50-2 17	1 63 (8)	0 92-2.74
Blood 30'-40' <sup>a</sup>	0.78 (4)	0 57-1.16	0 78 (8)	0 59-1 22
Liver	1 76 (4)	0 35-2 95	4 31 (10)	2 03 -7 28
Kidney	0 42 (4)	0 22-0 61	0 37 (10)	0 20 -0 61
Spleen	3 26 (4)	1 27-6 22	2 08 (10)	0 39 -5 36
Adrenals	0 53 (4)	0 32-0 73	0.68 (10)	0 26 -1 92
Thyroid	0 21 (3)	0 10-0 28	0 44 (8)	0 11 -1 50
Brain	0 03 (2)	0 03	0 04 (4)	0 03-0 05
Bile <sup>a</sup>	0 85 (4)	0 39-1 41	1 40 (7)	0 14-3 57
Lung	0 61 (2)	0 54-0 68	1 03 (5)	0 47-1 80
Leg muscle	0 08 (2)	0 06-0 10	0 09 (5)	0.007-0 20
Heart	0 30 (2)	0 24-0 35	0.30 (5)	0 16-0 38
Urine <sup>a</sup>	0 13 (4)	0 01-0 32	0 06 (8)	0 002-0 27

<sup>a</sup> Fluids, values given per cc<sup>b</sup> The number in the parentheses is the number of animals whose tissues were averaged

The use of labeled Congo red provided a means of confirming the observations of others regarding the urinary elimination of the dye. Harmon, *et al.* (11), reported urinary excretion following intravenous injection. Unger (1), on the basis of an accurate extraction procedure, found only a faint trace of the dye. Richardson (8) reported a slight undetermined amount in rabbit urine and none in cat urine. Reference to Table I shows that variable, small but discrete amounts of activity are eliminated via the urine. That this activity is not due to liberated  $I^{131}$  from metabolized dye is borne out by the fact that normal guinea pigs injected with an equal number of counts of  $NaI^{131}$  showed a urinary activity greater than any of the dye-injected normal or scorbutic animals. Additional confirmation that the activity in urine was not due to liberated  $I^{131}$  is borne out by the relatively low count in the thyroid glands of dye-injected guinea pigs compared with those animals injected with the same number of counts of  $I^{131}$ , in which the urine was higher than the average of all the experimental animals on labeled dye, and very many times higher than most. The thyroid count of the animals injected with  $I^{131}$  was 4 to 20 times higher than either the normal or scorbutic animals injected with labeled dye. Incidentally, reference to Table I shows that the thyroid of the scorbutic animals was twice that of the normals. Our results confirm Richardson (8), that a blood-brain barrier to the dye seems operative.

Histological examination of the kidney and liver samples of some of the scorbutic animals indicated very slight deposits of amyloid-like substance which took the specific stains.

## DISCUSSION

The use of trace-labeled Congo red permits physiological studies of the localization of the dye in amyloid tissue as well as its normal distribution and excretion. It was felt by the authors that one of the reasons that the tetraiodinated Congo red of Martin and Bang (2), who attempted to use this as a radiopaque substance, may have failed to localize sufficiently in amyloid tissue, was due to the changed physiological behavior of the heavily iodinated molecule. Our clearance rates from the blood are similar to those of Knorpp (9) whose congo red was only trace labeled with  $S^{35}$ .

The guinea pig was chosen for these experiments because it was felt that the induced chronic scurvy and resultant amyloidosis would make it an almost ideal test animal. However, it has been shown that only when the scurvy is prolonged is the formation and deposition of amyloid accomplished. Mice injected subcutaneously with solutions of sodium caseinate (12) over periods of several months develop amyloidosis and these are being prepared for confirmatory studies.

## SUMMARY

Two syntheses for the preparation of labeled Congo red containing  $I^{131}$  have been described. The identity and purity has been established by spectrophotometry, chromatography, and radioautography. The method of "direct iodination" was applied successfully to trypan blue (13). Studies were made of the blood disappearance, tissue distribution, and excretion in normal and scorbutic guinea pigs. The feasibility of external counting as a means of measuring blood disappearance and localization has been demonstrated. Some of the observations regarding the distribution of the dye have been confirmed.

## REFERENCES

- (1) Unger, P. N., Zuckerbrod, M., Beck, G. J., and Steele, J. M., *J. Clin. Invest.*, 27, 111 (1948).
- (2) Martin, C. F., and Bang, H., *THIS JOURNAL*, 37, 102 (1948).
- (3) Pirani, C. L., Bly, C. G., Sutherland, K., and Chereso, F., *Science*, 110, 145 (1950).
- (4) Catchpole, H. R., *A.M.A. Arch. Pathol.*, 10, 1010 (1956).
- (5) Kingsley, G. R., and Schaffert, R. R., "Standard Methods of Clinical Chemistry," Vol. 2, Academic Press Inc., New York, N. Y., 1958, p. 147.
- (6) Somers, G. F., and Whittet, T. D., *J. Pharm. and Pharmacol.*, 8, 1010 (1956).
- (7) Anderson, W. A. D., "Pathology," 3rd ed., C. V. Mosby Co., St. Louis, Mo., 1957, p. 72.
- (8) Richardson, A. P., *Am. J. Med. Sci.*, 198, 82 (1939).
- (9) Knorpp, C. T., Holt, F. J., and Korst, D. R., "Journal of Nuclear Medicine, Special Convention Issue," June 17, 1959.
- (10) Bennhold, H., *Deut. Arch. klin. Med.*, 142, 32 (1923).
- (11) Harmon, P. H., and Kernwein, G., *A.M.A. Arch. Internal Med.*, 70, 416 (1942).
- (12) Pirani, C. L., Dept. of Pathology, College of Medicine, Univ. of Illinois, Chicago, personal communication.
- (13) Casen, B., Dept. of Nuclear Medicine and Radiation Biology, Univ. of California Medical Center, Los Angeles, personal communication.



# An Investigation of *Coprinus atramentarius* for the Presence of Disulfiram\*

By J. K. WIER† and V. E. TYLER, Jr.

*Coprinus atramentarius* Fries was grown in surface culture on selected media. The quantity of mycelium formed was small, no carpophores were produced. Addition of a low concentration of disulfiram to cultures of the fungus had no apparent effect on the development of the organism, and the added disulfiram disappeared from the cultures within sixty days. Concentrated extracts of the media and mycelia of the cultures of the fungus, as well as extracts of naturally-occurring carpophores, were investigated for the presence of disulfiram by means of a paper partition chromatographic method. It is concluded that the disulfiram-like physiological activity which was exhibited by the naturally-occurring carpophores of *C. atramentarius* is due to some agent other than disulfiram.

IT HAS LONG been known that ingestion of the mushroom *Coprinus atramentarius* Fries and the subsequent ingestion of alcohol gives rise to physiological symptoms very similar to those of the alcohol disulfiram syndrome. Reports of *Coprinus*-alcohol poisoning are common in the mycological literature (1-5) and antedate considerably the discovery of the alcohol disulfiram syndrome. The remarkable similarity between the two types of poisoning has given rise to considerable speculation that disulfiram might exist in *C. atramentarius* (4).

In 1956 two Czechoslovakian investigators, J. Simandl and J. Franc (6), reported the isolation of disulfiram from *C. atramentarius*. They extracted autolyzed carpophores of the fungus with carbon tetrachloride in the cold, evaporated the carbon tetrachloride extract, and extracted the resulting residue with anhydrous methanol. Evaporation and recrystallization yielded a crystalline substance with a melting point of 70.5°, the melting point of disulfiram, and this substance had the properties of disulfiram when subjected to paper partition chromatographic procedures. They did not attempt a quantitative determination.

Since the physiological action of the combination of alcohol and *C. atramentarius* suggested the possible presence of disulfiram in that fungus, and since the presence of disulfiram in the fungus had been reported by Simandl and Franc, it seemed of interest to determine if quantities of the organism could be produced successfully in culture and if the fungus would produce disulfiram when so grown. The culture of *C. atramentarius* used to initiate this investigation was ob-

tained from the Centraalbureau voor Schimmelcultures, Baarn, Holland.

Although numerous hymenomycetes, including a number of *Coprinus* species, have been successfully grown in the laboratory, a recent review (7) revealed that the culture of *C. atramentarius* had been studied by only one investigator, C. H. Chow (8).

A number of media have been employed for culture of *Coprinus* species (9-13). Fresh horse dung or extract of fresh horse dung, alone or in combination with other nutrients, was the substrate most commonly used. Chow used fresh horse dung as well as other media in his cultures of *C. atramentarius*. He maintained only a few cultures of this fungus, as the spores germinated with difficulty and in low percentage, and he reported that the organism did not produce normal carpophores in artificial culture.

During the course of the present investigation it became obvious that *C. atramentarius* was not producing disulfiram when grown on the media employed, and it was decided to investigate the effect on the development of the fungus of adding disulfiram to the culture medium.

Locally-occurring carpophores of *C. atramentarius* were also investigated for presence of disulfiram. In the spring of 1957 a case of typical *Coprinus* alcohol poisoning had occurred in Seattle, Washington (14). The victim, R. Levin, a University of Washington student, obtained the mushrooms which he ate from the exact site as that from which the mushrooms employed in this investigation were obtained. The growth habit of *C. atramentarius* is such that all these carpophores may be presumed to have arisen from the same mycelial source. Identification of the naturally occurring carpophores of *C. atramentarius* employed in this investigation was made by Professor D. E. Stuntz, Department of Botany, University of Washington.

\* Received September 25, 1959 from the College of Pharmacy, University of Washington, Seattle 5.

† Abstracted from a dissertation submitted to the Graduate School of the University of Washington by J. K. Wier in partial fulfillment of the requirements for the degree of Master of Science.

## EXPERIMENTAL

Culture of the Organism.—Seven media were originally employed for culture of *C. atramentarius* on a small scale. These were either media used previously for culture of macrofungi by other investigators or modifications of such media made by the authors in an attempt to improve the development of *C. atramentarius*. The media employed were the following:

## Medium No 1

Mannitol . . . . .	2 0%
Casein hydrolysate . . . . .	1 0%
FeSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0 01%
Basic nutrient solution (15) . . . . .	10 0%
Distilled water . . . . .	q s

This medium was used for the culture of various strains of *Claviceps purpurea* (15), and it has recently been employed for the culture of *Panacolis campanulatus* by V E Tyler, Jr (16)

## Medium No 2

Glucose . . . . .	2 0%
Casein hydrolysate . . . . .	1 0%
FeSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0 01%
Basic nutrient solution (15) . . . . .	10 0%
Distilled water . . . . .	q s

## Medium No 3

Glucose . . . . .	2 0%
Casein hydrolysate . . . . .	1 0%
Yeast extract . . . . .	1 0%
FeSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0 01%
Basic nutrient solution (15) . . . . .	10 0%
Distilled water . . . . .	q s

## Medium No 4

This consisted of Medium No 3 to which thiamine hydrochloride (200 mcg /L) was added.

## Medium No 5 (Modified McCrea's medium)

Maltose . . . . .	1 25%
Peptone . . . . .	0 125%
Yeast extract . . . . .	1 25%
KH <sub>2</sub> PO <sub>4</sub> . . . . .	0 25%
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0 125%
Distilled water . . . . .	q s

This medium has been employed for the culture of *Claviceps purpurea* (17) and gave satisfactory development of *C. atramentarius* in slant tube cultures.

## Medium No 6 (Modess medium)

Glucose . . . . .	0 5%
Malt extract . . . . .	0 5%
KH <sub>2</sub> PO <sub>4</sub> . . . . .	0 05%
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0 05%
NH <sub>4</sub> Cl . . . . .	0 05%
FeCl <sub>3</sub> . . . . .	0 005%
Distilled water . . . . .	q s

This medium has been used by the Centraalbureau voor Schimmellecultures for the culture of *Boletus luridus* and *Amanita muscaria* (18)

## Medium No 7

Maltose . . . . .	0 5%
K <sub>2</sub> HPO <sub>4</sub> . . . . .	0 025%
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	0 05%
Ca(NO <sub>3</sub> ) <sub>2</sub> ·H <sub>2</sub> O . . . . .	0 05%
Horse dung extract . . . . .	33 33%
Distilled water . . . . .	q s

The horse dung extract was prepared by boiling 1 Kg of fresh horse dung with 1 L. of water for five minutes, straining, and filtering the product, and making to a volume of 1 L. with water. This medium was used by Bille-Hansen (12) for the culture of a number of *Coprinus* species.

For the small scale cultures, 125-ml portions of the various media were introduced into Roux-type culture flasks. These flasks of media were then sterilized by autoclaving, cooled, and inoculated with the fungus. The inoculated flasks were placed in a constant temperature cabinet maintained at 25° ± 1°. The cabinet had glass doors, and the cultures were exposed to the indirect artificial illumination of the room in which the cabinet was located. The most rapid growth of the organism occurred in medium No 7, the horse dung extract medium, and it was selected for routine use.

Subsequent cultures were maintained in both the Roux-type culture flasks and in diphtheria toxin culture bottles, the latter containing 1,200 ml of medium. These cultures were also kept in the constant temperature cabinet. All cultures were harvested when visible growth of mycelium had ceased, a period of sixty to ninety days.

The medium of each culture was separated from the mycelium by straining through a coarse mesh sieve and was then processed to determine the presence or absence of disulfiram. The mycelium so collected was placed in porcelain evaporating dishes and dried in a forced air oven at 45° for three days. The mycelia from cultures grown on the same medium and inoculated on the same day were combined and weighed. The dried mycelium was then investigated for presence of disulfiram.

The development of the fungus was very sparse in all the media. Table I presents a summary of data on the growth of the fungus in the seven media, and the ratios of weight of dried mycelium to volume of medium.

TABLE I—MYCELIAL YIELDS OF *Coprinus atramentarius* OBTAINED IN DIFFERENT MEDIA

Me- dium No	No of Cul- tures	Volume of Medium Processed, L	Total Weight Dried Mycelium, Gm	Dry Weight of Mycelium, mg /ml of Medium
1	12 <sup>a</sup>	1 500	0 99	0 66
2	8 <sup>a</sup>	1 000	0 75	0 75
3	8 <sup>a</sup>	1 000	0 82	0 82
4	8 <sup>a</sup>	1 000	0 64	0 64
5	16 <sup>a</sup>	2 000	1 47	0 74
6	8 <sup>a</sup>	1 000	0 65	0 65
7	19 <sup>a</sup>	2 325	2 40	1 03
7	13 <sup>b</sup>	15 600	12 05	0 77

<sup>a</sup> Roux flask cultures    <sup>b</sup> Diphtheria toxin flask cultures

To determine whether disulfiram had any effect upon the growth of *C. atramentarius*, twenty 250-ml. Erlenmeyer flasks containing 75 ml. of medium No. 7 to which 0 167 mg % of disulfiram had been added were inoculated with the fungus. The concentration of disulfiram which could be added was limited by its extremely low water solubility. Another series of 10 flasks of medium No. 7 to which no disulfiram was added was inoculated with the fungus.

An additional 10 flasks of medium No. 7 containing the disulfiram were prepared but not inoculated. The two series of flasks which had been inoculated with the fungus were harvested at the end of sixty days. The total weight of dried mycelium from the 20 cultures with added disulfiram was 2.55 Gm. (1.70 mg. mycelium per ml. of medium). The total weight of the dried mycelium from the 10 cultures with no added disulfiram was 1.30 Gm. (1.73 mg. mycelium per ml. of medium). The added disulfiram apparently had no effect on the development of the fungus.

Five of the 10 flasks of uninoculated medium containing added disulfiram were tested for the presence of that compound shortly after they were prepared. The other five flasks of medium in that series were similarly tested after sixty days. Disulfiram was detected in both cases. Apparently prolonged solution in the medium did not cause significant decomposition of the disulfiram. Neither the medium nor mycelium of the 20 cultures to which disulfiram had been added contained detectable quantities of disulfiram at the end of the sixty-day growth period. The fungus had apparently decomposed the added disulfiram.

When medium No. 7 which had been solidified by the addition of 2% agar was inoculated with the fungus, a sparse feathery growth of mycelium occurred. Small darkened areas of mycelial compaction occurred which exuded a small amount of liquid similar in appearance to the honeydew of ergot. No carpophores were produced.

**Detection of Disulfiram.**—Disulfiram was separated from semipurified extracts by paper partition chromatography and rendered visible on the sheets by treatment with a suitable reagent. An attempt to utilize the general procedure of Divatia, Hine, and Burbridge (19) for the spectrophotometric determination of disulfiram in blood proved unsuccessful when applied to disulfiram in medium No. 7.

The chromatographic procedure chosen was a modification of that employed by Simandl and Franc (6). Sheets of Whatman No. 1 filter paper, 22 cm. X 56 cm., were immersed for a few seconds in a mixture of kerosene and benzene (1 to 9). The sheets were allowed to air-dry for five minutes and were then reimmersed in the kerosene-benzene mixture. After another five minute drying period, spots of the solutions to be investigated were applied along a line 12 cm. from one end of the kerosene-impregnated sheets of paper. Total amounts of solution varying from 0.25 to 0.50 ml. were applied to the spots in successive 10- to 20- $\mu$ L. portions. Each portion was allowed to dry thoroughly before the next was applied. The spotted sheets were then placed in a glass chromatographic chamber, and the chromatograms were formed in the descending direction with a solvent system composed of 65% ethanol in water. The atmosphere of the chamber was allowed to equilibrate with the solvent for twenty-four hours prior to the introduction of the chromatograms. After drying, the chromatograms were sprayed with a saturated aqueous solution of cuprous chloride. As this reagent dried, greenish-yellow spots appeared where disulfiram was present. After spraying, these spots appeared dark purple under an ultraviolet lamp, but they did not fluoresce.

In this system, the  $R_f$  values obtained for disul-

fram ranged from 0.70 to 0.74. Tailing was not objectionable, and the area of the disulfiram spot at the end of its travel was no more than two to three times the area of the original spot. A 100-mcg. quantity of disulfiram applied to the paper could be detected readily after formation of the chromatogram.

Each of the concentrated extracts obtained from medium, mycelium, or carpophores was applied to the sheets in amounts of 0.25 and 0.50 ml. Each extract was also applied in a mixed spot with a 150-mcg. quantity of known disulfiram. Control spots of disulfiram<sup>1</sup> were also applied to each sheet.

**Extraction Procedures.**—The media of all cultures in a given series, i.e., those cultures of the same medium which had been inoculated on the same day, were combined after harvesting the mycelium. Each L. of medium was subjected to four successive extractions of five minutes duration with 200-ml. portions of chloroform. The four chloroform extracts were combined and allowed to evaporate spontaneously. The small amount of residue was redissolved in 5 ml. of chloroform per L. of medium and tested for presence of disulfiram.

The dried mycelium from each series of cultures was ground to a moderately coarse powder in a mortar, packed into a micropercolator, and extracted exhaustively with chloroform. This percolate was allowed to evaporate spontaneously, and the resulting residue was redissolved in 2 ml. of chloroform per Gm. of mycelium extracted. This concentrated extract was then tested for presence of disulfiram. No disulfiram was detected in either the medium or the mycelium of any of the cultures.

Carpophores of naturally-occurring *C. atramentarius* arising from the same mycelial source as those which had caused typical *Coprinus*-alcohol poisoning in R. Levin were investigated for the presence of disulfiram. A total fresh weight of 6.1 Kg. of mushrooms was processed by a method similar to that reported by Simandl and Franc (6). The mushrooms were allowed to autolyze for two days and were then reduced to a mash in a Waring Blendor. This mash was subjected to four successive extractions of five minutes duration with 300 ml. of carbon tetrachloride per Kg. of fresh mushrooms. The solids were separated from the solvent after each extraction by centrifugation. The combined carbon tetrachloride extracts were filtered and allowed to evaporate spontaneously. The fatty residue remaining was mixed with clean sand, packed into a glass percolator, and extracted exhaustively with anhydrous methanol. The methanol percolate was evaporated to dryness under vacuum at 30°. The slight gummy residue obtained in this way was redissolved in 10 ml. of anhydrous methanol per Kg. of fresh carpophores processed. No crystalline material was obtained. These extracts were purified by chromatographing in the manner previously described and eluting with anhydrous methanol the area which corresponded to the area where known disulfiram appeared on the same chromatograms. These purified extracts were then rechromatographed. No disulfiram was found in any of the carpophore extracts.

<sup>1</sup> Authentic disulfiram supplied through the courtesy of Ayerst Laboratories, New York, N. Y.

In order to ascertain if the extraction procedures which were employed would actually recover disulfiram, a quantity of that compound was added to a quantity of naturally-occurring carpophores of *C. atramentarius*. An 800-Gm. quantity of fresh mushrooms was reduced to a mash in a Waring Blendor, and 0.01% of disulfiram was added to one-half of the mash. After two days both portions of the mash were processed. The portion to which no disulfiram was added gave no test for disulfiram. The portion to which disulfiram was added was extracted, and the residue was dissolved in 10 ml. of anhydrous methanol. Fifty microliters of this concentrated extract were chromatographed and gave a color reaction with saturated cuprous chloride solution of greater intensity than 100 mcg. of known disulfiram. This indicated that the 10 ml of extract contained over 20 mg. of disulfiram. The extraction procedure was therefore more than 50% efficient.

### DISCUSSION

The quantities of mycelium produced by *C. atramentarius* when cultured on the seven media employed were extremely small. Medium No. 7, the horse dung extract medium, gave a slightly higher yield of the fungus than any of the other media tested. The small darkened areas of mycelial compaction which were observed when the fungus was grown on solid medium were similar to the structures described by C. H. Chow (8) as rudimentary carpophores; however, no true carpophores were produced.

The processes of extraction employed were shown to have an efficiency of at least 50%, and the chromatographic technique which was used could readily detect a 100-mcg. quantity of disulfiram. Consequently, the extracts of the culture media must have contained less than 0.2 mg. % of disulfiram or that compound would have been detected. Similarly, the mycelium extracts must have contained less than 80 mg. % of disulfiram, and the extracts of the naturally-occurring carpophores must have contained less than 0.4 mg. % of disulfiram (fresh weight basis). If disulfiram were present in the carpophores at a level just below that which was detectable by the procedures employed, approximately 125 Kg. of fresh mushrooms would have to be consumed in order to obtain the ordinary daily maintenance dose of 500 mg. of disulfiram (20). *C. atramentarius* arising from the same mycelial source as that investigated by the authors is known to possess disulfiram-like physiological activity when eaten in

ordinary amounts. As the maximum possible level of disulfiram in the carpophores investigated by the authors is far below that which could exert physiological activity, it must be concluded that the disulfiram-like activity of these carpophores is due to some other agent present in the fungus.

The fact that disulfiram did not exist in the *C. atramentarius* investigated does not entirely rule out the possibility that a recoverable concentration of that compound did occur in the fungus investigated by Simandl and Franc (6). Such variation could be due to several factors. First, the positive identification of the black-spored *Agaricus* is extremely difficult. One cannot be positive that the "*Coprinus atramentarius*" investigated by Simandl and Franc is identical with the organism investigated here. However, such a discrepancy is rather unlikely. Second, nutritional and environmental factors can alter the metabolism of organisms in such a way as to change, at least quantitatively, the products of that metabolism. Third, various genetic strains of *C. atramentarius* may exist which differ appreciably in their metabolism. The features upon which the taxonomy of the *Coprinini* is based are chiefly morphological and anatomical in nature. Very little is known of the genetics of these organisms, and more than one genotype may well exist within a single phenotype.

### REFERENCES

- (1) Ramsbottom, J., "Mushrooms and Toadstools," new ed., Collins, London, 1954, p. 55.
- (2) Smith, A. H., "Mushrooms in Their Natural Habitats," Sawyer's Inc., Portland, Ore., 1949, pp. 128, 574.
- (3) Pilát, A., "Mushrooms," H. W. Blj, Amsterdam, 1954, pp. 65-67.
- (4) Josseland, M., *Mycologia*, 44, 829(1952).
- (5) Chiffot, M. J., *Bull. Soc. Mycol. France*, 32, 63(1916).
- (6) Simandl, J., and Franc, J., *Chem. listy*, 50, 1862 (1956).
- (7) Quintanilha, A., and Pinto-Lopes, J., *Bol. Soc. Bot. 24*, 115(1950).
- (8) Chow, C. H., *Botanist*, 26, 89(1934).
- (9) Mounce, I., *Brit. Mycol. Soc. Trans.*, 7, 198(1921).
- (10) Lutz, M. L., *Bull. Soc. Mycol. France*, 41, 310(1925).
- (11) Kuhner, R., *ibid.*, 62, 1925.
- (12) Bille-Hansen, E., *Bot.*
- (13) Bille-Hansen, E., *Phys.*
- (14) Wier, J. K., Dissertation, University of Washington, 1959, pp. 2-3.
- (15) Tyler, V. E., Jr., and Schwarting, A. E., *This Journal*, 41, 590(1952).
- (16) Tyler, V. E., Jr., unpublished data from an investigation of *Panaeolus campanulatus*, 1959.
- (17) Schwarting, A. E., and Hiner, L. D., *This Journal*, 34, 11(1945).
- (18) Centraalbureau voor Schimmelteculures, Baarn, Holland, personal communication, 1957.
- (19) Divatia, K. J., Hine, C. H., and Burbridge, T. N., *J. Lab. Clin. Med.*, 39, 974(1952).
- (20) Goodman, L. S., and Gilman, A., "The Pharmacological Basis of Therapeutics," 2nd ed., The Macmillan Co., New York, N. Y., 1955, pp. 116-120.

# Interaction of Preservatives With Macromolecules IV\*

## Binding of Quaternary Ammonium Compounds by Nonionic Agents

By PATRICK P. DELUCA† and H. B. KOSTENBAUDER

Equilibrium dialysis studies utilizing a semi-permeable nylon membrane indicate a high degree of association and accompanying inhibition of quaternary ammonium germicides such as cetylpyridinium chloride and benzalkonium chloride with nonionic surfactants such as Tween 80. Cetylpyridinium chloride was also found to bind to methylcellulose, but not to PVP or Polyox. Benzalkonium chloride was not bound to methylcellulose, PVP, or Polyox under the conditions of this study.

ALTHOUGH SEVERAL investigators have presented microbiological data indicating that nonionic surface-active agents can interfere with the activity of cationic germicides such as the quaternary ammonium compounds, textbooks and reference works generally do not emphasize the high degree of inactivation which can sometimes occur in these systems. It has been suggested that the observed inactivation is attributable to a preferential association or binding of the cationic agent with the nonionic surfactant (1), although no previous data have been published which would indicate the magnitude of this association. The present work was undertaken to obtain quantitative data for the degree of binding of several cationic agents by some typical nonionics and to compare the degree of binding with the degree of inactivation of the cationics.

Phospholipids such as lecithin have long been popular as neutralizing or inactivating media for quaternary ammonium compounds in germicidal testing (2-7). Quisno, Gibby, and Foter suggested the use of an inactivating medium consisting of lecithin and Tween 80, indicating that the lecithin was the primary inactivator with the Tween acting as a dispersing agent (8). In 1949 Gershenfeld and Stedman (9) reported their observations on the activity of several cationics, including cetylpyridinium chloride and cetyltrimethylammonium bromide, in the presence of varying concentrations of a nonionic surfactant, noting enhancement of activity at low surfactant concentration and inhibition at higher surfactant concentrations. Davies (10) reported that a polyethylene glycol cetostearyl ether was

capable of neutralizing the bacteriostatic effect of cetyltrimethylammonium bromide and Ritter (11) indicated that Tween 80 was capable of neutralizing the bactericidal effect of cetylpyridinium chloride on *tubercle bacilli*. Barr and Tice (12) investigated seven quaternary ammonium compounds in the presence of 5% polyoxyethylene 20 sorbitan monostearate and found that only benzalkonium chloride was effective in a concentration of 0.1%. Wedderburn (13) indicated that benzalkonium chloride also was subject to inactivation by nonionics, including sucrose ester surfactants, under the conditions of her studies. Moore and Hardwick (1) presented microbiological data on combinations of quaternary ammonium compounds and nonionic surfactants and devoted considerable discussion to the relative effectiveness of such combinations. Other reports of inactivation are discussed in the recent review by Beckett and Robinson (14).

Studies cited above (1, 9) indicate that in very dilute solutions of the nonionic surfactants there is an enhancement of the effectiveness of the quaternary ammonium compound, but at higher concentrations of the surfactant the activity of the germicide is greatly diminished. This is typical of the behavior of many germicides in the presence of surface-active agents (1, 15, 16).

In the present study two typical quaternary ammonium compounds, cetylpyridinium chloride and benzalkonium chloride, were selected to illustrate the relative magnitude of any interaction which might occur between these agents and nonionics such as Tween 80, methylcellulose, polyvinylpyrrolidone, and high molecular weight ethylene oxide polymers. The particular quaternary ammonium compounds used in this study were selected principally because both agents show absorption in the ultraviolet and thus can readily be determined spectrophotometrically.

### EXPERIMENTAL

**Reagents.**—Cetylpyridinium chloride (1-hexadecylpyridinium chloride);<sup>1</sup> benzalkonium chloride U. S. P. (a mixture of alkyldimethylbenzylammonium chlorides in which the alkyls range from  $C_8H_{17}$  to  $C_{18}H_{37}$ );<sup>2</sup> cetyldimethylbenzylammonium

\* Received August 21, 1959, from the School of Pharmacy, Temple University, Philadelphia, Pa. Revised January 1960.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Walter G. Karr Fellow, Smith Kline & French Laboratories, Philadelphia, Pa.

<sup>1</sup> Ceepryn Chloride, The Wm. S. Merrell Co., Cincinnati, Ohio.

<sup>2</sup> Zephiran Chloride, Winthrop-Stearns Inc., New York, N. Y.

chloride,<sup>3</sup> Tween 80,<sup>4</sup> a commercial sample and also a sample passed through an ion exchange column, methylcellulose, 15 c p s,<sup>5</sup> polyvinylpyrrolidone,<sup>6</sup> Polyoxy WSR-35.<sup>7</sup>

To assure that any observed inactivation of quaternary ammonium compounds by Tween 80 would not be attributable to the presence of unesterified oleic acid in the Tween sample, the Tween solution was passed through a mixed-bed ion exchange column<sup>8</sup> to remove traces of oleic acid which might be present. Quantitative removal of oleic acid was established by adding known amounts of free oleic acid to Tween 80 samples and determining oleic acid content by potentiometric titration with approximately 0.1 *N* NaOH before and after passage through the ion exchange resin. Subsequent studies indicated that the contribution of any traces of oleic acid in the commercial sample of Tween 80 to the inactivation of quaternary ammonium compounds was probably insignificant.

Before the polyvinylpyrrolidone was employed in the dialysis studies, the sample was extracted with ether in a Soxhlet extractor for forty eight hours.

**Conductivity Measurements.**—To establish approximate critical micelle concentrations for the samples of cetylpyridinium chloride and cetyltrimethylbenzylammonium chloride used in this study, conductivity measurements were obtained for aqueous solutions at room temperature (23–25°). An Industrial Instruments model RC M15 conductivity bridge and a dipping type conductivity cell with a cell constant of approximately 100 cm<sup>-1</sup> were employed. The water used was that used throughout the study and had a specific conductance of approximately  $2 \times 10^{-5}$  ohm<sup>-1</sup>.

**Dialysis Studies.**—Dialysis membranes employed in the studies involving Tween 80 were nylon membranes as described previously by Patel and Kostenbauder (18). These membranes were previously shown to be impermeable to Tween 80 (18). For dialysis studies involving methylcellulose, polyvinylpyrrolidone, and Polyoxy, Visking cellulose casings were employed. The general procedure for these studies consisted of placing inside the dialysis bag 20 ml of a solution containing the quaternary ammonium compound and, after tightly knotting the open end, placing the bag into a 125 ml bottle containing 40 ml of Tween or other polymer solution. A polyethylene film and a screw cap were employed as the closure, and the bottles were rotated at 9 r p m in a constant temperature bath at 30°. As might be expected for the long chain quaternary ammonium compounds, equilibration times of five to seven days were

necessary for cetylpyridinium chloride solutions and two to three days for benzalkonium chloride solutions. The presence of Tween 80, however, reduced the equilibration time for cetylpyridinium chloride to one to two days.

After equilibration, aliquots were removed from both sides of the membrane and concentrations of quaternary ammonium compounds were determined spectrophotometrically at a wavelength of 259 mμ for cetylpyridinium chloride and 261.5 mμ for benzalkonium chloride, using a Beckman DU spectrophotometer. Any interference due to the macromolecule was eliminated by using the appropriate concentration in the reference cell.

**Microbiological Studies.**—Observations were made on the influence of several nonionic surface-active agents on the bacteriostatic activity of cetylpyridinium chloride and benzalkonium chloride, using *Aerobacter aerogenes*,<sup>9</sup> a Gram-negative organism. The methods and culture medium previously described by Pisano and Kostenbauder (19) were employed. Approximate inhibitory concentrations for cetylpyridinium chloride and benzalkonium chloride in the presence of Tween 80, Triton X-100,<sup>10</sup> and Pluronic L62,<sup>11</sup> were obtained through visual observation of samples each day for a period of two weeks.

## RESULTS

**Critical Micelle Concentration for Cationics.**—Approximate critical micelle concentrations for cetylpyridinium chloride and cetyltrimethylbenzylammonium chloride (CDBAC) were obtained from the conductivity data illustrated in Fig. 1. The critical micelle concentration for cetylpyridinium chloride was found to be approximately  $1.0 \times 10^{-3}$  *M*, while that for the CDBAC was found to be approximately  $1.0 \times 10^{-4}$  *M*.

**Tween 80.**—Cetylpyridinium chloride was found to interact to an extremely high degree with Tween 80 when the cationic was employed in concentrations comparable to those which might be encountered in product formulations. Figures 2 and 3 illustrate typical adsorption isotherms for this system, and in Fig. 4 these data are presented in a manner which indicates that in an aqueous system containing Tween 80 and cetylpyridinium chloride, the ratio of total to free cetylpyridinium chloride is primarily a function of the concentration of Tween 80. As illustrated in a previous publication (19), the ratio of total to free germicide can often be employed to predict required preservative concentrations in the presence of the Tween. Figure 4 indicates that at a concentration of 1% Tween 80, approximately 95% of the cetylpyridinium chloride present is bound to the Tween and thus inactivated. Even at a concentration of 0.1% Tween, the data suggest that approximately 60% of the total cetylpyridinium chloride would be inactivated. The dialysis studies indicated no significant difference in binding of cetylpyridinium chloride by a commercial sample of Tween 80 and a sample passed through an ion exchange resin to remove any oleic acid present.

<sup>3</sup> Sample obtained through the courtesy of Dr. George D. Wessinger, Sterling Winthrop Research Institute, Rensselaer, N. Y.

<sup>4</sup> Tween 80 is polyoxyethylene 20 sorbitan monooleate, Atlas Powder Co., Wilmington, Del.

<sup>5</sup> Methocel, 15 c p s, the Dow Chemical Co., Midland, Mich.

<sup>6</sup> Pladone, Antara Chemicals Division of General Aniline and Film Corp., New York, N. Y.

<sup>7</sup> Polyoxy WSR 35 is a poly(ethylene oxide) of exceptionally high molecular weight (17), Union Carbide Chemicals Co., New York, N. Y.

<sup>8</sup> The ion exchange column contained a mixture of the strongly basic quaternary amine ion exchange resin Amberlite IRA 400, previously treated with NaOH solution and the cation exchange resin Amberlite IR 120 previously treated with HCl solution.

<sup>9</sup> ATCC No. 8308.

<sup>10</sup> Triton X 100 is an alkylaryl polyether alcohol, Rohm and Haas Co., Philadelphia, Pa.

<sup>11</sup> Pluronic L62 is a polyoxyethylene polyoxypropylene surfactant, Wyandotte Chemicals Corp., Wyandotte, Mich.

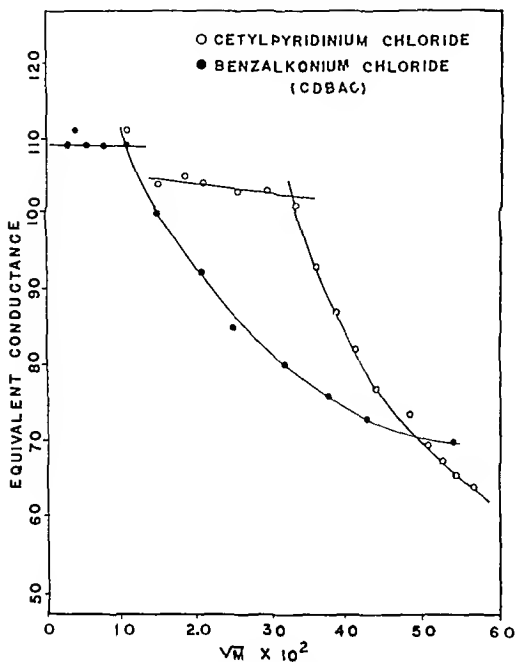


Fig. 1.—Determination of critical micelle concentration for cetylpyridinium chloride and cetyldimethylbenzylammonium chloride through conductivity measurements, 23–25°. Critical micelle concentrations were approximately  $1.0 \times 10^{-3} M$  for cetylpyridinium chloride and  $1.0 \times 10^{-4} M$  for cetyldimethylbenzylammonium chloride.

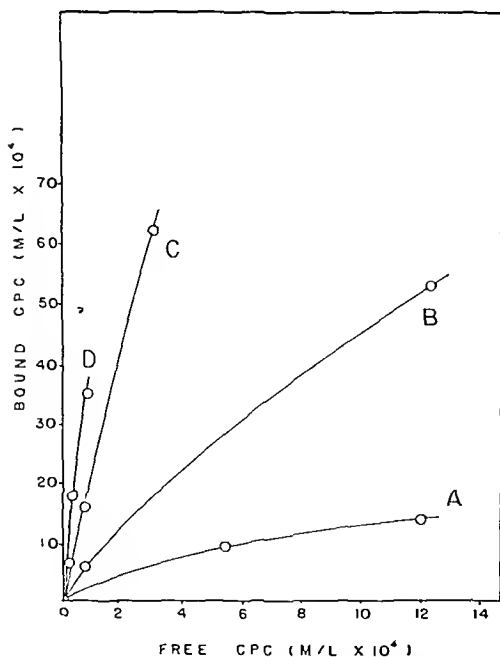


Fig. 2.—Adsorption isotherms for binding of cetylpyridinium chloride by Tween 80 in aqueous solution at 30°. A, 0.1%; B, 0.5%; C, 1.0%; D, 2.5% TW 80.

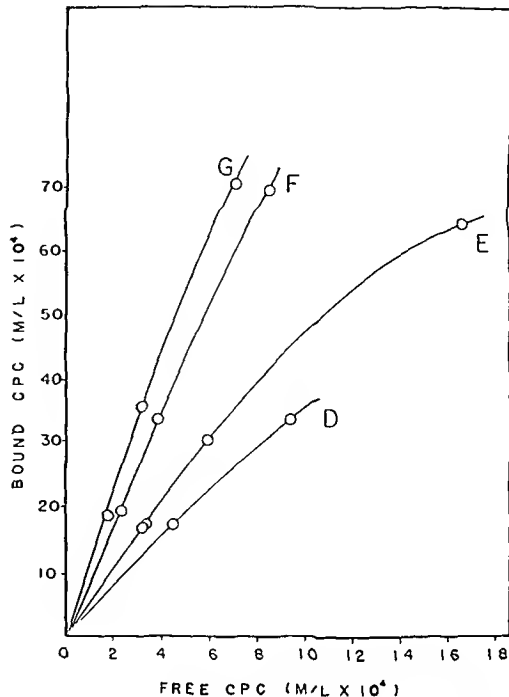


Fig. 3.—Adsorption isotherms for binding of cetylpyridinium chloride by Tween 80 in aqueous solution at 30°. D, 2.5%; E, 5.0%; F, 7.5%; G, 10.0% TW 80.

The marked scatter of points in Fig. 4 is partly a result of magnification of experimental error due to the pronounced binding which occurs. Calculation of  $r$ , the ratio of total to free preservative, in some cases involves dividing the total concentration by a number smaller by a factor of approximately 100; thus, a small error in either total or free concentration is greatly magnified in calculation of the  $r$  value. Despite the inherent inaccuracy of such treatment of data, the convenience of a graph such as that presented in Fig. 4 justifies its presentation.

Data for the binding of benzalkonium by Tween 80 are illustrated in Fig. 5. While the binding of benzalkonium chloride is considerably less than that exhibited by cetylpyridinium chloride, it nevertheless is sufficient to cause inactivation. At a concentration of 1% Tween 80, approximately 50% of the benzalkonium chloride present is bound to the Tween. There appeared to be no significant difference in the degree of binding exhibited by benzalkonium chloride U. S. P., which is described as consisting of alkyls in the range  $C_8 - C_{18}$  (20), and the binding shown by a pure sample with a  $C_{16}$  chain, i. e., CDBAC.

Figure 6 represents adsorption isotherms for the interaction of cetylpyridinium chloride and CDBAC with a very dilute solution of Tween 80. This study was included to permit observations on the degree of binding in the presence of free quaternary concentrations both above and below the normal critical micelle concentration for the quaternary compound. These isotherms differ markedly from those obtained in higher concentrations of Tween

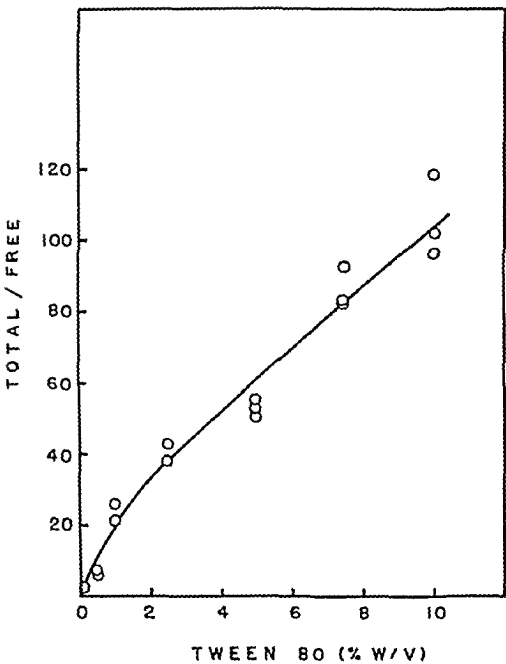


Fig. 4.—The data illustrated in Figs. 2 and 3 plotted to give the ratio,  $r$ , of total to free cetylpyridinium chloride as a function of the concentration of Tween 80.

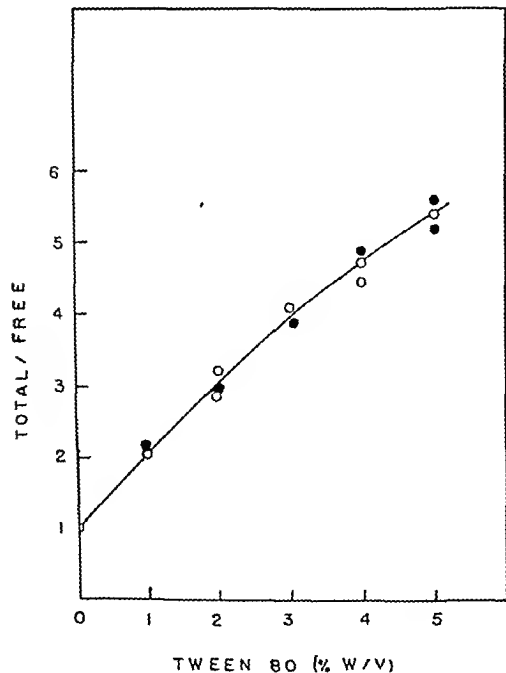


Fig. 5.—The ratio,  $r$ , of total to free benzalkonium chloride as a function of Tween 80 concentration at 30°. Total benzalkonium chloride concentration 0.025–0.10%. O, benzalkonium chloride U. S. P.; ●, cetyltrimethylbenzylammonium chloride.

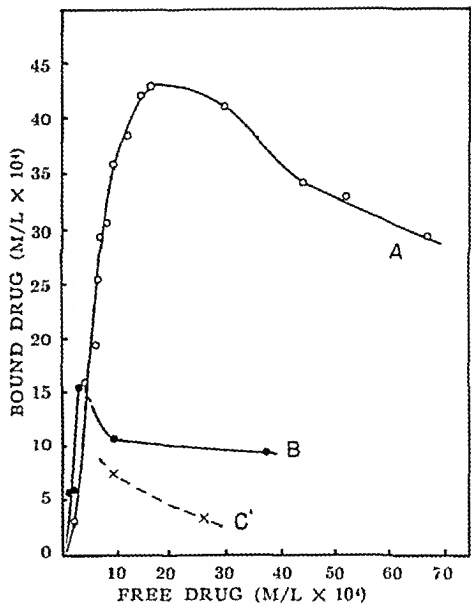


Fig. 6.—Adsorption isotherms for binding of cetylpyridinium chloride and cetyltrimethylbenzylammonium chloride in 0.2% Tween 80 at 30°. These curves illustrate the degree of binding occurring at concentrations of the quaternary ammonium compounds both above and below their normal critical micelle concentrations. A, cetylpyridinium Cl; B, benzalkonium Cl(CDBAC); C, cetylpyridinium Cl, 0.05  $M$  NaCl. Curve C illustrates the influence of electrolyte on the degree of interaction.

80, showing a maximum in the adsorption isotherm in the region of the critical micelle concentration.

Methylcellulose.—Figure 7 shows the interaction of cetylpyridinium chloride with methylcellulose. The binding is highly dependent on the cetylpyridinium chloride concentration, passing through a maximum in the region of the normal critical micelle concentration for cetylpyridinium chloride. Benzalkonium chloride was found to exhibit no detectable binding to methylcellulose up to a concentration of 2% methylcellulose and 0.125% cationic.

Figure 8 shows the influence of pH and electrolyte on the interaction of cetylpyridinium chloride with methylcellulose. The binding appears to be increased somewhat in the presence of strong base, but is suppressed by 0.05  $M$  NaCl and almost eliminated by 0.05  $M$  HCl.

Polyvinylpyrrolidone.—Neither cetylpyridinium chloride nor benzalkonium chloride was found to interact significantly with polyvinylpyrrolidone at a concentration of 2% polyvinylpyrrolidone and 0.08% quaternary ammonium compounds.

Polyox.—Neither cetylpyridinium chloride nor benzalkonium chloride was found to interact significantly with this polymer at concentrations of 0.2% Polyox and 0.07% quaternary ammonium compound.

Microbiological Studies.—Concentrations of cetylpyridinium chloride and benzalkonium chloride required to inhibit growth of *Aerobacter aerogenes* in the presence of several nonionic surfactants are



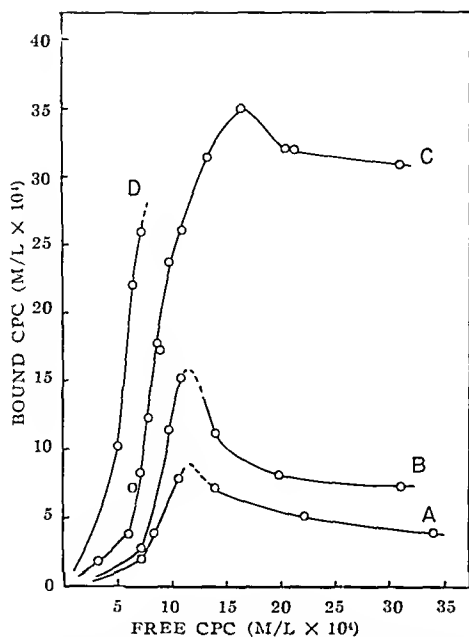


Fig. 7.—Binding of cetylpyridinium chloride by methylcellulose at 30°. A, 0.05%; B, 0.1%; C, 0.5%; D, 1.0% methylcellulose.

presented in Table I. These studies were made with polymer solutions which were passed through ion exchange resins to remove any fatty acid or electrolyte present and also with some untreated samples of Tween 80. No attempt was made to determine precise inhibitory concentrations, but the increased concentrations of quaternary ammonium compound indicate a somewhat higher degree of inactivation than would be predicted from a consideration of Figs. 4 and 5.

As would be predicted from the binding studies, benzalkonium chloride was found to be inhibited to a lesser degree than was the cetylpyridinium chloride. This observation is in agreement with that of Barr and Tiee (12), who found that in the presence of Tween 60 benzalkonium chloride was effective in a concentration of 0.1% while cetylpyridinium chloride was not.

The results obtained with Pluronic L62 and Triton X-100 indicate that these agents show an ability to inactivate the quaternary ammonium compounds quite similar to that exhibited by Tween 80.

## DISCUSSION

**Tween 80.**—The formation of mixed micelles of quaternary ammonium compound and nonionic surfactant has been suggested as a possible mechanism for the association with molecules such as Tween 80 (1). For such an interaction, the degree of binding would be expected to increase with increasing length of the hydrocarbon chain of the cationic, at least so long as the concentration of the free cationic did not exceed the normal critical micelle concentration. The critical micelle concentrations for the cetylpyridinium chloride and CDBAC used in these studies were found to be approximately  $1.0 \times 10^{-3}$  M and  $1.0 \times 10^{-4}$  M, respectively. Only in the

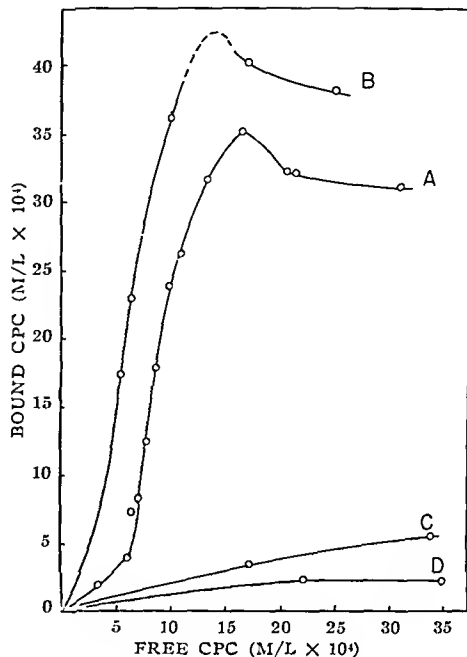


Fig. 8.—Influence of pH and electrolyte on the interaction of cetylpyridinium chloride with 0.5% methylcellulose in aqueous solution at 30°. A, distilled water; B, 0.003 M NaOH; C, 0.05 M NaCl; D, 0.05 M HCl.

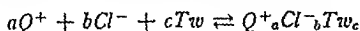
TABLE I.—INFLUENCE OF SEVERAL NONIONIC SURFACTANTS ON CONCENTRATIONS OF CATIONIC REQUIRED TO INHIBIT *Aerobacter aerogenes*

Nonionic	Inhibitory Concentration	
	Cetylpyridinium Cl	Benzalkonium Cl
0	1-100,000 to 1-250,000	No growth at 1-100,000
0.5% Tween 80 <sup>a</sup>	1-2,500 to 1-5,000	....
2.0% Tween 80 <sup>a</sup>	1-250 to 1-500	....
3.0% Tween 80 <sup>b</sup>	1-100 to 1-250	1-500 to 1-1,000
3.0% Triton X-100 <sup>b</sup>	1-100 to 1-250	....
3.0% Pluronic L62 <sup>b</sup>	1-500 to 1-1,000	....

<sup>a</sup> Commercial sample. <sup>b</sup> Treated with ion exchange resin before use.

concentrations of Tween below 0.5% were free cetylpyridinium concentrations of this magnitude approached, although in all cases the concentration of free benzalkonium was in excess of the critical micelle concentration. It was at first suspected that the marked difference in degree of binding of cetylpyridinium chloride and benzalkonium chloride by the Tween might be a result of heterogeneity in the composition of the alkyl of the benzalkonium chloride, since the U. S. P. specifies C<sub>8</sub>-C<sub>18</sub> for this compound. However, subsequent binding studies using a pure sample of cetyldimethylbenzylammonium chloride (CDBAC) resulted in data which exactly duplicated the degree of binding exhibited by the U. S. P. product.

If the interaction between the quaternary ammonium compound and the Tween can be visualized as an equilibrium between mixed micelles of quaternary ammonium-Tween and individual quaternary ammonium ion and halide ion



the interaction would be expected to be dependent on the concentration<sup>12</sup> of the long chain cation according to the law of mass action.<sup>13</sup>

$$K' = \frac{(Q^+_a Cl^-_b Tw_c)}{(Q^+)^a (Cl^-)^b (Tw)^c} \quad (\text{Eq. 1})$$

In Eq. 1,  $K'$  is an equilibrium constant,  $(Tw)$  is the free Tween concentration,  $(Q^+)$  is the concentration of individual long chain cation,  $(Cl^-)$  is the concentration of the individual halide ion, and  $(Q^+_a Cl^-_b Tw_c)$  is the concentration of the mixed micelle or complex.

Several investigators have observed that in an aqueous solution of an ionic surfactant the concentration of the individual long chain ion passes through a maximum as the total concentration of surfactant in the solution is increased. This maximum occurs in the concentration range at which micelle formation is observed. The existence of the maximum can be shown by the application of the law of mass action to micelle formation, the most extensive treatments being the work of Mysels (21) and of Sexsmith and White (22, 23). For the system  $aQ^+ + bX^- \rightleftharpoons Q^+_a X^-_b$  where

$$K'' = \frac{(Q^+_a X^-_b)}{(Q^+)^a (X^-)^b} \quad (\text{Eq. 2})$$

Sexsmith and White, by solving for  $(Q^+)$  as a function of total quaternary concentration,  $(C)$ , and setting  $d(Q^+)/d(C) = 0$ , demonstrated that for several model quaternary ammonium surfactants the concentration of individual quaternary ammonium ion,  $(Q^+)$ , passes through a maximum if  $a > b \geq 2$ . The concentration of the individual halide ion,  $(X^-)$ , and the ion product of quaternary ammonium ion and halide ion, however, continue to increase as the total quaternary ammonium concentration is increased.

The existence of this maximum in individual quaternary ammonium ion concentration provides a probable explanation for the observed differences in binding affinities of cetylpyridinium chloride and CDBAC. If the concentration of individual quaternary ammonium ion parallels the total free quaternary ammonium compound concentration until micelles begin to appear, it might be expected that the approximately tenfold difference in critical micelle concentration for the cetylpyridinium chloride and CDBAC would result in a maximum attainable concentration of individual quaternary ammonium ion greater by approximately a factor of 10 for the cetylpyridinium chloride solutions. Occurrence of a maximum in individual quaternary am-

monium ion at extremely low concentrations of CDBAC suggests that the concentration of single ions of cetyldimethylbenzylammonium never approaches the level encountered in cetylpyridinium solutions. For an interaction between a quaternary ammonium compound and Tween 80, such as that illustrated in Eq. 1, a tenfold difference in quaternary ion concentration would be expected to exert a considerable influence on the degree of binding occurring.

If the proposed mechanism is operative, it should be possible to demonstrate maxima in the adsorption isotherms for the interaction of cetylpyridinium chloride and CDBAC with Tween 80. To this end, the binding data illustrated in Fig. 6 were obtained, using a very dilute solution of Tween 80. The existence and relative positions of the maxima in the adsorption isotherms are very clearly illustrated. These data indicate that the interaction is dependent on the concentration of the single quaternary ammonium ion and could not be attributed solely to the adsorption of ion pairs or micelles, since both the product of  $(Q^+)(Cl^-)$  and the concentration of micelles are known to increase steadily as the total quaternary concentration increases (23). The decrease in the degree of interaction at higher quaternary concentrations is most satisfactorily explained thermodynamically on the basis of a decrease in concentration of the individual quaternary ion.

Observations on the interaction of Tween 80 and cetylpyridinium chloride in the presence of 0.05 *M* NaCl are indicated by the broken line in Fig. 6. These points suggest a shift of the maximum in the adsorption isotherm to lower concentrations, and suggest that the presence of electrolyte might be a rather significant factor in determining the degree of binding.

The binding of organic electrolytes by nonionic surfactants is not limited to quaternary ammonium compounds; drugs such as chlorpromazine, promethazine, and tetracaine hydrochlorides are also bound, as are dyes and some anionic detergents (24).

**Methylcellulose.**—Available data for the binding of cations to cellulose derivatives pertain principally to those agents used in dyeing textiles. Numerous theories have been presented to account for the binding of dyes and metal ions to cellulose, some of which include: (a) ionic binding to residual carboxyls or to acidic hydroxyls by ion exchange, (b) ion-dipole interactions of cations with ethers or hydroxyls, (c) dipole-dipole interactions between hydroxyls of cellulose and polar groups of the dye molecule, (d) interactions between hydrocarbon portions of the adsorbate and the linear cellulose molecules.

More recent discussions seem to indicate that more than one mechanism is involved in most cases of adsorption to cellulose (22). Until recently the importance of the hydrophobic interaction was not fully recognized. It now appears to have been established that for a dye to have maximum affinity for a cellulose substrate the dye should be a long, planar molecule which can attain close approach to the linear cellulose chain. Where this condition is met there is an extremely high degree of stability for the dye-cellulose combination; the stability being attributed to interaction of the hydrophobic groups (25).

<sup>12</sup> Here the assumption is made that concentrations may be substituted for activities.

<sup>13</sup> The interaction would, of course, also be dependent on the concentration of the Tween and the halide ion, but these considerations are not vital to the discussion which follows.

Figure 7 illustrates the binding of cetylpyridinium chloride by methylcellulose as a function of methylcellulose and cetylpyridinium chloride concentration. The interaction shows a high dependency on cetylpyridinium concentration and the initial portion of the curve, showing a rapidly increasing slope, is similar in appearance to the binding of phenol by PVP and polyethylene glycols (26) and to the binding of phenol (27, 28) and organic ions (29, 30, 31) by proteins. Curves such as this are sometimes considered to result when adsorption of a critical quantity of drug results in a change in configuration of tightly coiled polymer molecules to make additional binding sites available. A curve of this nature, which seemingly indicates limitless binding capacity, is also similar to multilayer adsorption isotherms, and in this case might be explained on the basis of possible tail-to-tail adsorption of quaternary after the initial adsorption of a critical number of ions. A mechanism of this nature has recently been demonstrated for the binding of cationic dyes and surfactants by cotton, viscose rayon, and oxycellulose (22, 23, 32, 33). These workers demonstrated that the ion exchange process involved residual carboxyls in the cellulose.

A recent report by Fishman and Miller (34) indicates that starch also interacts with quaternary ammonium compounds, and from the data presented the mechanism for the interaction would appear to be quite similar to that which occurs with cellulose and methylcellulose.

Data presented in Fig. 8 illustrate the effect of pH and electrolyte on the degree of binding, and the results are indicative of an ion exchange mechanism as the initial step in the interaction. The interaction is apparently enhanced by dilute alkali, greatly diminished by 0.05 *M* NaCl, and almost entirely eliminated by 0.05 *M* HCl.

The maximum which appears in the adsorption isotherm occurs in the neighborhood of the critical micelle concentration for cetylpyridinium chloride and is typical of adsorption isotherms described by Sexsmith, *et al.* (22, 23, 32, 33), for the interaction of quaternary ammonium compounds with cellulose. Sexsmith, *et al.*, demonstrated that the existence of the maximum can be attributed to the occurrence of a maximum in the concentration of individual quaternary ion and the resultant maximum in the ion exchange adsorption (22, 23).

Failure of benzalkonium chloride to interact with methylcellulose can be attributed to the relatively low concentration of individual quaternary ion which can occur in these solutions. Failure of Polyox and PVP to interact with the quaternary ammonium compounds can probably be attributed to the nonionic and nonionogenic nature of these polymers.

**Microbiological Observations.**—The estimation of inhibitory concentrations of cationic agents in the presence of the nonionics indicated an even higher degree of inactivation of the cationic than would be predicted from the equilibrium dialysis studies. This discrepancy can probably be attributed to the presence of approximately 0.1 *M* total electrolyte in the culture medium. Addition of electrolyte is known to lower the critical micelle concentration for quaternary ammonium compounds (35), and at concentrations below the critical micelle concentration the presence of electrolyte would also be

expected to enhance the interaction between the cationic and the Tween. While an exact correlation between binding data and microbiological data would apparently require knowledge of the interaction as a function of electrolyte concentration, the dialysis studies presented here do verify the existence of a remarkably high degree of interaction for some quaternary ammonium germicides with nonionics such as Tween 80 and methylcellulose, and illustrate the perils of making the general assumption that nonionic agents are compatible with cationic and anionic drugs.

## SUMMARY

1. Equilibrium dialysis studies indicate an extremely high degree of association and accompanying inactivation of quaternary ammonium germicides in the presence of nonionic surface-active agents. In 1 per cent aqueous solutions of Tween 80 approximately 95 per cent of the total cetylpyridinium chloride or 50 per cent of the total benzalkonium chloride present would be bound to the Tween and thus inactivated. In 0.1 per cent Tween 80 solution approximately 60 per cent of the cetylpyridinium chloride would be bound.

2. Methylcellulose was found to interact significantly with cetylpyridinium chloride, but not with benzalkonium chloride. Polyvinylpyrrolidone and Polyox were not found to interact with these cationics.

3. These studies indicate quite clearly that it is not justifiable to assume that nonionic agents are always compatible with cationic and anionic drugs.

## REFERENCES

- (1) Moore, C. D., and Hardwick, R. B., *Mfg. Chemist*, 27, 305(1956); 29, 194(1958).
- (2) Baker, Z., Harrison, R. W., and Miller, B. F., *J. Exptl. Med.*, 74, 621(1941).
- (3) Mueller, W. S., Seeley, D. B., and Larkin, E. P., *Soap Sanit. Chemicals*, 23, 123(1947).
- (4) Rahn, O., *THIS JOURNAL*, 36, 134(1947).
- (5) Lawrence, C. A., "Surface Active Quaternary Ammonium Germicides," Academic Press, Inc., New York, N. Y., 1950, pp. 126 ff.
- (6) Reddish, G. F., "Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization," 2nd ed., Lea & Febiger, Philadelphia, Pa., 1957, p. 51.
- (7) Schwartz, A. M., Perry, J. W., and Berch, J., "Surface Active Agents and Detergents," Vol. 11, Interscience Publishers, Inc., New York, N. Y., 1958, p. 208.
- (8) Quisno, R., Gibby, I. W., and Foter, M. J., *Am. J. Pharm.*, 118, 320(1946).
- (9) Gershenfeld, L., and Stedman, R. L., *ibid.*, 121, 249(1949).
- (10) Davies, G. E., *J. Hyg.*, 47, 271(1949).
- (11) Ritter, H. W., *Appl. Microbiol.*, 4, 114(1956).
- (12) Barr, M., and Tice, L. F., *THIS JOURNAL*, 46, 44(1957).
- (13) Wedderburn, D. L., *J. Soc. Cosmetic Chemists*, 9, 211(1958).
- (14) Beckett, A. H., and Robinson, A. E., *Soap, Perfumery & Cosmetics*, 31, 454(1958).
- (15) Bean, H. S., and Berry, H., *J. Pharm. and Pharmacol.*, 3, 639(1951).
- (16) Alawala, N. A., and Riegelman, S., *THIS JOURNAL*, 42, 267(1953).
- (17) Bailey, F. E., Jr., Powell, G. M., and Smith, K. L., *Ind. Eng. Chem.*, 50, 8(1958).
- (18) Patel, N. K., and Kostenbauder, H. B., *THIS JOURNAL*, 47, 289(1958).

- (19) Pisano, F. D., and Kostenbauder, H. B., *ibid*, 48, 310(1959)  
 (20) "United States Pharmacopeia," 15th Rev., Mack Printing Co., Easton, Pa., 1955, p. 83  
 (21) Mysels, K. J., *J. Colloid Sci.*, 10, 507(1955)  
 (22) Sexsmith, F. H., Ph. D. Dissertation, Princeton University, 1956  
 (23) Sexsmith, F. H., and White, H. J., Jr., *J. Colloid Sci.*, 14, 630(1959)  
 (24) Hurwitz, A. R., DeLuca, P. P., and Kostenbauder, H. B., To be published  
 (25) Allingham, M. M., Giles, C. H., and Neustadter, E. L., *Discussions Faraday Soc.*, 16, 92(1954)  
 (26) Guttman, D., and Higuchi, T., *THIS JOURNAL*, 45, 659(1958)  
 (27) Cooper, E., and Sanders, E., *J. Phys. Chem.*, 31, 1 (1927).

- (28) Cooper, E., and Woodhouse, D., *Biochem. J.*, 17, 600 (1923)  
 (29) Karush, F., *J. Phys. Chem.*, 56, 70(1952)  
 (30) Few, A. V., Ottewill, R. H., and Parreira, H. C., *Biochim. et Biophys. Acta*, 18, 136(1955)  
 (31) Hill, R. M., and Briggs, D. R., *J. Am. Chem. Soc.*, 78, 1590(1956)  
 (32) Sexsmith, F. H., and White, H. J., Jr., *J. Colloid Sci.*, 14, 598(1959)  
 (33) Gotshal, Y., Rebenfeld, L., and White, H. J., Jr., *ibid*, 14, 619(1959)  
 (34) Fishman, M. M., and Miller, R., Paper Presented before Division of Carbohydrate Chemistry, 136th Meeting, American Chemical Society, Atlantic City, N. J., September 1959  
 (35) Westwell, A. E., and Anacker, E. W., *J. Phys. Chem.*, 63, 1022(1959)

## Urinary Excretion Kinetics for Evaluation of Drug Absorption III\*

### Method for Calculation of Absorption Rate and Application to Tetracycline Absorption in Humans

By EINO NELSON

A method was developed to calculate drug absorption rate from urinary excretion rate measurements and applied to study tetracycline absorption in humans after oral ingestion of 25, 50, 100, and 200-mg. doses. Calculation of absorption rate indicated that this drug was absorbed at a rate of about 100 to 140 mg./hr., one hour after 200-mg. doses, and at about 20 to 30 mg./hr., one hour later. Rates at these times decreased when the dose was reduced. Wide individual variation in absorption rate was observed among members of the test panel.

THERE ARE relatively few methods available that can be applied to study drug absorption quantitatively in the intact animal after drug ingestion orally. The method of Dominguez (1) may be applied, but its use requires frequent blood samples at short intervals of time after drug ingestion. The intubation method of Nicholson and Chornock (2) allows accurate measurement, but is an uncomfortable procedure to the test subject. The present work describes a method that is based on data collected from urinary excretion measurements. The method is applicable to all routes of drug administration, but is applied here to the oral route to study the mechanism of tetracycline's absorption in humans.

#### THEORY AND DEVELOPMENT OF THE METHOD FOR ABSORPTION RATE CALCULATION

The method is based on writing a material balance accounting for drug at absorption sites, in the body, and eliminated drug in excreted urine or removed

by other processes. Several assumptions must be made and conditions met in order to make the method experimentally and mathematically tractable. These conditions and assumptions are (a) A significant amount of drug must be excreted unchanged in the urine. While there can be no arbitrarily set lower limit on the percentage eliminated by this route, it is desirable that this percentage be at least of the order of 30. Error in rate calculation increases markedly with error in percentage when the latter is a low value. (b) It is necessary to assume that drug in circulation is in equilibrium with other fluids of distribution at all times even though there is always a delay in the attainment of this equilibrium. (c) After this equilibrium is reached, the drug must disappear from the blood stream by an apparent first-order process which is the usual case.

At any time the dose of a drug taken can be accounted for by the following expression

$$A_d = A_b + A'_e + A_i \quad (\text{Eq. 1})$$

In Eq. 1,  $A_d$  is the dose,  $A_b$  is the amount in the body in blood and other fluids of distribution,  $A'_e$  is the amount eliminated by urinary excretion and other processes, and  $A_i$  is the amount of drug remaining at the absorption sites. It follows on differentiation of Eq. 1 with respect to time and rearrangement that

$$-dA_d/dt = dA_b/dt + dA'_e/dt \quad (\text{Eq. 2})$$

\* Received September 19, 1958, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

Supported in part by a Grant in aid from the Squibb Institute for Therapeutic Research, New Brunswick, N. J.

In Eq. 2,  $t$  is time,  $dA_i/dt$  is the rate of disappearance of drug from the absorption sites,  $dA_b/dt$  is the rate of change of amount of drug in the body, and  $dA'_e/dt$  the rate of elimination from the body.

The rate of elimination may be deduced from excretion data. This rate is equal to the sum of the urinary excretion rate,  $dA_e/dt$  and the rate of elimination by other processes,  $dA''_e/dt$ , i. e.

$$dA'_e/dt = dA_e/dt + dA''_e/dt \quad (\text{Eq. 3})$$

As a consequence of the considerations discussed by Dominguez and Pomerene (3) and later by Hough (4), a simple relationship exists between the two righthand terms of Eq. 3. These terms are, respectively, equal to  $k_1A_b$  and  $k_2A_b$  where  $k_1$  and  $k_2$  are the rate constants in reciprocal hours for each process. Hence

$$dA'_e/dt = A_b(k_1 + k_2) \quad (\text{Eq. 4})$$

It helps to define,  $K = k_1 + k_2$ , where  $K$  is the rate constant that describes the rate of removal by the combined processes. Also in accord with the considerations discussed by Hough (4), the rate of excretion in the urine may be described by the following

$$dA_e/dt = KfA_b \quad (\text{Eq. 5})$$

where  $f$  is the fraction of drug reaching circulation that is eliminated unchanged in the urine. Solving Eq. 5 for  $A_b$  and substituting in Eq. 4 yields

$$dA'_e/dt = (1/f)(dA_e/dt) \quad (\text{Eq. 6})$$

which when substituted in Eq. 2 gives

$$-dA_b/dt = dA_b/dt + (1/f)(dA_e/dt) \quad (\text{Eq. 7})$$

The term,  $dA_b/dt$ , in Eqs. 2 and 7 may be found from Eq. 5. Taking the derivative of Eq. 5 and solving for  $dA_b/dt$  obtained by this process and substituting for its value in Eq. 7 yields

$$-dA_b/dt = (1/Kf)(d^2A_e/dt^2) + (1/f)(dA_e/dt) \quad (\text{Eq. 8})$$

Since absorption rate is the same as disappearance rate only with opposite sign, Eq. 8 may be stated as

$$\text{Absorption rate} = (1/Kf)(d^2A_e/dt^2) + (1/f)(dA_e/dt) \quad (\text{Eq. 9})$$

The rate terms in Eq. 9 may be obtained graphically or by use of polynomials fitted to excretion data as will be described later. The value of  $K$  for a given drug may be found from either the literature or from the linear portion of a plot of the logarithm of excretion rate *vs.* time, where excretion rate may be either the graphically determined values or values obtained from the amount excreted in a given time divided by the time interval over which the sample was collected. The value of  $K$  is easily found from such a plot by noting the time necessary for a 50% decrease in rate and making use of the relationship,  $K = 0.693/t_{1/2}$ , where  $t_{1/2}$  is the half life noted.

Calculation of rate of absorption by Eq. 9 is independent of the size of the dose given or the percentage of this dose absorbed. In fact, if for example, the same drug is given in several different forms such as different salts, calculation of absorption rate gives the absorability of each form.

**Collection of Data.**—Collection of excretion data from a given drug for use in the application of Eq. 9 requires some consideration of excretion velocity in order to obtain meaningful results. When the cumulative amount excreted *vs.* time curve is constructed, based on data obtained after administration of drug by other than intravenous means, it always shows an inflection point. To characterize properly the excretion curve, at least two urine collections should be made before the appearance of this inflection point and at least two afterwards. Spacing of urine collections will depend on the rapidity with which the drug is absorbed and excreted. The experimental data in this report indicate collection period spacing to some extent.

**Comparison with Other Methods of Calculating Absorption Rate.**—The only other methods available to calculate absorption rate are those in which unabsorbed material is recovered from experimental animals (2), or the method applied by Dominguez (1) to creatinine. The method of Dominguez (1) requires the assumption that drug in blood and other fluids of distribution is in equilibrium at all times as is the case in the method presented here. However, the present method does not require information on the total volume of distribution of drug which is required in the Dominguez method. The determination of this quantity is subject to some error.

The approximate method for calculating absorption rate presented here is generally applicable under the conditions stated and has been presented in some detail. Use of the method does not depend on the site of drug administration, although it is applied here to absorption from the gastrointestinal tract.

## EXPERIMENTAL PROCEDURE

**Test Subjects.**—The test panel consisted of five adults in apparent good health. Their ages and weights are given in the first entry of Table I.

**Dosage Forms.**—Tetracycline hydrochloride was given in the form of particles with an average diameter of 100  $\mu$  contained in hard gelatin capsules mixed with 200 mg. sodium bicarbonate. The particles and dosage forms were prepared as previously described (5). The tetracycline hydrochloride was of commercial purity without additives. Four sets of dosage forms, each set containing 25, 50, 100, and 200 mg. tetracycline hydrochloride, respectively, were used in the tests. The salt and dosage form used was to insure that absorption would not be rate-limited by solution rate (5).

**Conduction of Tests and Assay.**—The drug was taken on fasting stomachs in the morning under conditions which have been previously described (5). The dosage for a given subject on a given test day was randomly selected.

Following ingestion, subjects collected urine specimens at one, two, three, and four hours, and aliquots of these, as well as aliquots of preingestion specimens, were assayed microbiologically for tetracycline hydrochloride activity by the cup plate method (6).<sup>1</sup> The tetracycline hydrochloride standard for comparison was prepared from the same batch used to prepare the dosage forms.

<sup>1</sup> The author is indebted to Mrs. Yoko Yuzuriha for conduction of these assays.

TABLE I.—CUMULATIVE MG TETRACYCLINE HYDROCHLORIDE AND ML URINE EXCRETED TO VARIOUS TIMES IN HOURS<sup>a</sup>

Subject <i>b</i>	mg Excreted				ml Urine			
	1 0	2 0	3 0	4 0	1 0	2 0	3 0	4 0
25-mg Dose Tetracycline HCl								
E (39-77)	0 8	1 7	2 7	3 9	340	400	500	870
S (33-57)	0 7	2 1	3 4	4 2	80	150	220	260
N (21-77)	0 2	1 7	4 0	5 1	260	460	530	600
C (29-84)	0 1	0 5	0 8	1 0	70	170	230	280
J (26-107)	0 2	0 6	1 2	1 8	60	130	190	260
Mean	0 4	1 3	2 4	3 2	162	262	334	454
50-mg Dose Tetracycline HCl								
E	1 8	5 1	7 8	9 8	110	190	460	645
S	1 3	3 4	5 9	7 8	90	200	350	460
N	0 4	3 9	8 1	10 5	110	210	270	320
C	0	1 1	5 4	9 3	80	140	180	210
J	1 4	5 7	8 9	11 5	80	170	250	310
Mean	1 0	3 8	7 2	9 8	94	182	302	389
100-mg Dose Tetracycline HCl								
E	0	1 8	6 3	9 9	210	360	680	1140
S	1 8	6 2	9 8	13 2	70	150	240	450
N	1 0	6 0	13 0	18 6	40	140	330	430
C	1 9	11 9	17 9	26 6	60	110	180	250
J	1 9	9 7	15 2	22 4	210	400	540	710
Mean	1 3	7 1	12 4	18 1	118	232	394	596
200-mg Dose Tetracycline HCl								
E	2 8	10 3	18 7	24 7	250	570	670	790
S	2 4	10 2	21 1	29 5	270	510	590	790
N	3 8	16 0	25 0	31 0	70	150	190	230
C	0 6	7 3	15 8	27 5	110	240	350	410
J	1 8	6 8	13 0	16 9	40	80	140	230
Mean	2 3	10 1	18 7	25 9	148	310	388	490

<sup>a</sup> All values in terms of the activity of tetracycline hydrochloride<sup>b</sup> Quantities in parentheses are subject's age followed by his weight in Kg

## RESULTS AND DISCUSSION

**Tetracycline Excretion.**—Table I summarizes excretion data with pertinent related information. In almost all cases shown the excretion values listed were from a collection at the indicated times. In the few cases where urine collections could not be made exactly on the hour, individual plots of cumulative amounts excreted *vs* time curves were constructed and excretion values for entry in Table I determined by interpolation. The mean values on Table I are plotted on Fig. 1.

**Fitting Experimental Data to Polynomials in Powers of Time.**—The mean values for amounts of tetracycline excreted from the several dosage schemes shown in Table I were fitted to polynomials in powers of time of the form

$$Ae = at + bt^2 + ct^3 + dt^4 \quad (\text{Eq } 10)$$

The individual data from 200-mg doses of the drug were also fitted to equations of this form. The values of the coefficients of Eq 10 are given in Table II for the combined excretion from various doses. Table III lists the coefficients of Eq 10 as obtained by using the individual data of test subjects after they ingested 200-mg doses.

The first and second derivatives of Eq 10 with coefficients evaluated were used to supply these respective terms needed in Eq. 9. After dividing

the value of the second derivative by *K*, it was then possible to describe absorption rate in terms of its product with *f*. This quantity was then calculated at one and two hours for each of the several doses.

**Illustrative Example.**—Fitting the mean values of tetracycline excreted in one, two, three, and four hours from 200-mg doses gave the following equation which corresponded to Eq 10

$$Ae = -2.65t + 6.26t^2 - 1.41t^3 + 0.104t^4 \quad (\text{Eq } 11)$$

The derivative of Eq 11 has the value

$$dAe/dt = -2.65 + 12.5t - 4.23t^2 + 0.416t^3 \quad (\text{Eq } 12)$$

The second derivative has the value

$$d^2Ae/dt^2 = 12.5 - 8.46t + 1.25t^2 \quad (\text{Eq } 13)$$

The value of Eq 13 at one and two hours after multiplying by the reciprocal of *K*, 11.5 (7) is 60.8 and 6.70, respectively. The value of Eq. 12 at the same times is 6.04 and 8.78, respectively. Adding the one hour values just calculated in accordance with Eq. 9, absorption rate expressed as a product with *f* was about 70 mg *f*/hour. At two hours the value was about 15 mg *f*/hour, indicating absorption had almost ceased. With tetracycline, *f* may be estimated to be between 0.5 and 0.7 meaning absorption rate at one hour was between

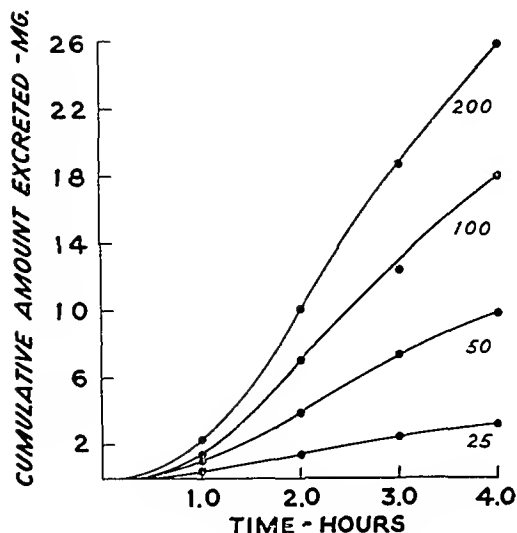


Fig 1—Variation in cumulative amounts of tetracycline excreted with time from several doses of tetracycline hydrochloride. Each curve labeled with the amount of tetracycline hydrochloride taken in mg.

TABLE II—COEFFICIENTS OF THE POLYNOMIAL EQUATION (EQ 10) FOR VARIOUS DOSES OF TETRACYCLINE HCl<sup>a</sup>

Dose mg	a	b	c	d
25	0 100	0 308	0	0
50	-0 252	1 41	-0 151	0
100	-4 10	7 46	-2 31	0 246
200	-2 65	6 26	-1 41	0 104

<sup>a</sup> Entries of zero means less than 0.01

TABLE III—COEFFICIENTS OF THE POLYNOMIAL EQUATION (EQ 10) FOR EACH TEST SUBJECT TAKING 200-MG DOSES OF TETRACYCLINE HCl

Subject	a	b	c	d
E	-0 942	4 48	-0 759	0 021
S	-0 249	2 35	0 436	-0 137
N	-7 23	15 43	-4 89	0 492
C	-5 31	7 81	-2 14	0 237
J	-0 091	1 91	0 044	-0 063

100 to 140 mg /hour and at two hours between 20 to 30 mg /hour, when 200-mg doses were taken.

Summary of Data From Various Doses.—The calculated absorption rate as a product with  $f$  after the various doses is summarized in Table IV. The same quantity for each test subject after 200-mg doses were ingested by them is listed in Table V.

TABLE IV—VALUES OF ABSORPTION RATE AT ONE AND TWO HOURS<sup>a,b</sup>

Time, hr	Dose				
	25	50	100	200	
1	10	25	52	70	
2	10	15	0	15	

<sup>a</sup> Expressed as a product with  $f$  <sup>b</sup> To the nearest 5 mg  $f$ /hr

TABLE V—VALUES OF ABSORPTION RATE FOR EACH TEST SUBJECT FOLLOWING 200-MG DOSES OF TETRACYCLINE HCl<sup>a,b</sup>

Time, hr	G	S	N	C	J	Mean
1	60	70	100	70	40	70
2	20	50	0	20	20	20

<sup>a</sup> Expressed as a product with  $f$  <sup>b</sup> To the nearest 5 mg  $f$ /hr

It is apparent that wide variation existed in the absorbability of tetracycline hydrochloride by the test subject, but this variation is not unusual in biological experiments. Comparison of the absorption rate values from pooled data following 200-mg. doses (Table IV) to the means of the individually calculated absorption rates (Table V) following the same dose of drug tends to indicate that the averaging of data did not cause excessive error in subsequent mathematical analysis.

No practical alternative procedure to the present method exists to study the absorption mechanism of tetracycline in humans in the range of doses given here. The low tetracycline serum levels resulting from the smaller doses precluded application of the method of Dominguez (1). Analysis for tetracycline in the urine was possible from the smaller doses because the volume of fluid in which excreted material was contained was much smaller than the volume of fluid in which the drug was distributed in the body.

Calculations with Other Drugs.—A comparison of absorption rates of sulfacthyliadiazole calculated by the method described here and by the Dominguez method (1) has been made (8).

## REFERENCES

- (1) Dominguez, R., Goldblatt, H., and Pomerene, E., *Am J Physiol*, 119, 429(1937)
- (2) Nicholson, J. T. L., and Chornock, F. W., *J Clin Invest*, 21, 505(1942)
- (3) Dominguez, R., and Pomerene, E., *Proc Soc Exptl Biol Med*, 60, 173(1945)
- (4) Hough, J., *Nature*, 179, 100(1957)
- (5) Nelson, E., *THIS JOURNAL*, 48, 96(1959)
- (6) Grove, D. C., and Randall, W. A., "Assay Methods of Antibiotics. A Laboratory Manual," Medical Encyclopedia, Inc., New York, N. Y., 1955, p. 50
- (7) Unpublished work conducted in this laboratory.
- (8) Nelson, E., and Schaldemose, I., *THIS JOURNAL*, 48, 489(1959)

# The Relationship of Metal Binding to the Biological Activities of Phenethylbiguanide\*

By EUGENE D. WEINBERG, ROBERT CHERNIN, and JOHN H. BILLMAN

Phenethylbiguanide has been found to form metal complexes with divalent copper and nickel; on the basis of infrared absorption spectra and on theoretical considerations, a revised structure for the metal complexes of biguanides is proposed. Antimicrobial activities of phenethylbiguanide are not affected by divalent copper and nickel but are significantly depressed by iron. The relationship of these findings to the biological mechanisms of action of the compound is discussed.

SUCH POWERFUL metal binding agents as 8-hydroxyquinoline, hematoxylin, and certain thiosemicarbazones are diabetogenic (1, 2). Chemical modification of 8-hydroxyquinoline to prevent metal binding is accompanied by a loss of the ability to cause diabetes (3, 4). It has been suggested that the metal binding compounds act by altering the availability of zinc to the islet cells although the necessity of zinc for either

the formation, storage, or functioning of insulin has by no means been established (1, 5, 6).

Certain other strong metal binding agents as isoniazid and the tetracyclines are not diabetogenic, at least at the chemotherapeutic levels ordinarily attained; nor do they appear to affect carbohydrate metabolism in any way (7, 8). Still other metal binding agents, such as the salicylates, are not only nondiabetogenic but can actually reduce the fasting blood sugar and glycosuria of moderately severe diabetic patients (9). A group of metal binding agents that have long been known to possess hypoglycemic activity for diabetic patients are certain diguanidines of which Synthalin A is the outstanding example (Fig. 1). An additional group of hypoglycemic compounds with potential metal binding ability includes 1-(*p*-chlorophenyl)-4-isopropyl biguanide (Paludrine), amylbiguanide, and  $\beta$ -phenethylbiguanide (PEBG, DBI). Of the biguanides, PEBG (Fig. 1) has been the most intensively tested for possible use as an oral antidiabetic drug.

The ability of PEBG to form specific metal chelates has not been reported. This paper describes studies of (a) the metal binding activity of PEBG and (b) the effects of metallic ions on certain antimicrobial properties of this compound

## CHEMICAL STUDIES

Aqueous 0.05 *M* solutions of PEBG·HCl<sup>1</sup> were combined with ammoniacal 0.05 *M* to 1.0 *M* solutions of the nitrates of lithium, beryllium, sodium, magnesium, aluminum, potassium, calcium, chromium, manganese, ferrous and ferric iron, cobalt, nickel, divalent copper, zinc, strontium, silver, cadmium, tin, cesium, barium, monovalent and divalent mercury, and lead. In each case, the pH reaction was adjusted to 6-7 with 1.0 *M* NH<sub>4</sub>OH, and a blank without PEBG was included to eliminate the possibility that any precipitates that might form might be metal hydroxides rather than metal chelates

Precipitates of PEBG·HCl plus the inorganic nitrates were obtained only with the salts of copper and nickel. Crystals of the copper and nickel complexes of PEBG were subsequently prepared by digesting the freshly prepared metal hydroxides with PEBG·HCl in a water bath, followed by concentrating and cooling the filtrate. The crystals of the copper and nickel complexes were blue-violet

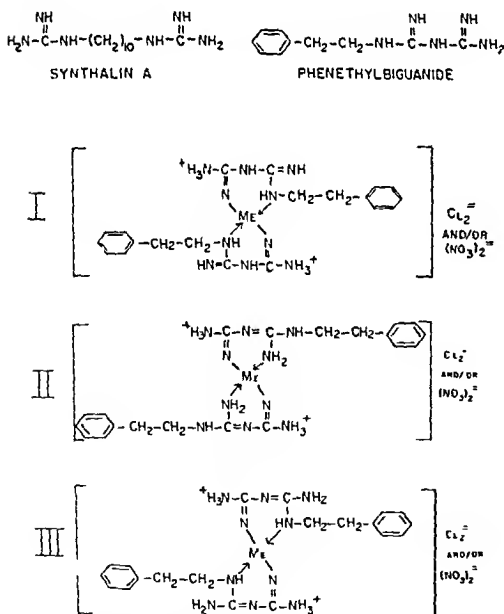


Fig. 1.—Structural formulas of Synthalin A, phenethylbiguanide, and theoretical structural formulas of the metal chelates of phenethylbiguanide.

\* Received September 3, 1959, from the Departments of Bacteriology and Chemistry, Indiana University, Bloomington.

Technical assistance of Misses Judith Brooks, Janet Bowers, and Sharon Nance is acknowledged.

This project was supported in part by Grant E-2252 from the U. S. Public Health Service.

<sup>1</sup> Phenethylbiguanide was kindly supplied by the U. S. Vitamin Corp through the courtesy of Dr. H. S. Sadow.



and pink, respectively. The infrared absorption spectra of a KBr mull of PEBG and the copper and nickel complexes were obtained with an Infracord spectrophotometer with a slit width of  $25\ \mu$  (Fig. 2)

Biguanide sulfate has been utilized for the determination of divalent copper and nickel with which it forms sparingly-soluble inner metallic complexes of the second order. Such ions as those of zinc, cadmium, magnesium, and the alkali metals do not interfere with this reaction (10). The copper and nickel phenylbiguanides have been described, as have also the metaphenylene, ethylene,  $\alpha$ -naphthyl, and *p*-phenethylbiguanide chelates of these metals (11, 12). The  $\alpha$ -copper complex of phenylbiguanide has been reported to be bluish-violet and the  $\beta$ -copper complex to be brick red. The  $\alpha$ -nickel complex is brick red and the  $\beta$ -nickel complex light yellow. By color analogy, the PEBG chelates prepared in the present study are probably the  $\alpha$ -copper form and the  $\beta$ -nickel form.

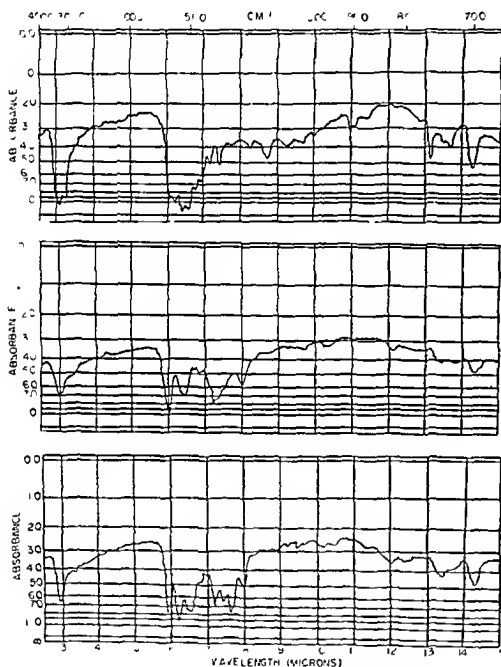


Fig. 2.—Infrared absorption spectra of phenethylbiguanide (top), copper chelate (middle), and nickel chelate (bottom).

According to the studies reported with phenylbiguanide (11), the structures of the PEBG chelates would be represented by I in Fig. 1. However, two additional possible structures are represented by II and III

It may be noted that the chelate rings in structure I contain only one double bond and that the second double bond is exocyclic. In contrast, the double bonds in structures II and III are both endocyclic. Inasmuch as six-membered chelate rings containing two double bonds are more stable than rings with one endocyclic and one exocyclic double bond, it is considered that structures II or III are more probable than I. The phenethyl group should make the

nitrogen in the adjacent amino group more basic than the nitrogen in the imino group and it would therefore be more likely that the former would participate in chelation than the latter. Accordingly, it is suggested that structure III would be more probable than structure II. Unfortunately, the infrared absorption spectra do not indicate which of the chelate structures has actually formed.

## MICROBIOLOGICAL STUDIES

Strains of *Serratia*, *Escherichia*, *Bacillus*, *Staphylococcus*, *Saccharomyces*, and *Chlorella* were grown in both complex and minimal synthetic agar media and the microstatic concentrations of PEBG were determined for each strain in each environment. Filter paper disks containing  $1 \times 10^{-3}$  M solutions of the respective nitrates listed in the section on chemical studies were placed on inoculated complex and synthetic agar media containing submicrostatic, microstatic, and excess microstatic concentrations of PEBG. In subsequent experiments the copper and nickel complexes of PEBG were tested for microstatic activity.

The minimal microstatic concentrations of PEBG in complex media and the effects of the nitrates of iron on these concentrations are given in Table I. It may be observed that with each microorganism, the activity of PEBG was depressed by iron. No significant depressing or enhancing effects on PEBG were observed with any of the other metallic nitrates tested except that of a slight depressing effect by aluminum with *Escherichia*. Similar minimal microstatic concentrations and depressing effects of iron were observed when the microorganisms were grown in synthetic media. The copper and nickel chelates possessed approximately the same microstatic activity as PEBG in complex media but were five to ten times more active in synthetic media. Presumably the chelates are dissociated by the microorganisms since the apparently enhanced activity in synthetic media was found to be caused by copper or nickel when these metals were tested (as the nitrates) in the absence of PEBG. These metallic ions are not toxic in complex media because of the numerous protective metal binding agents (amino acids, vitamins, etc.) in such media.

Molar concentrations of PEBG between  $1 \times 10^{-7}$  and  $1 \times 10^{-3}$  were tested for microstatic activity to determine the possible existence with this compound of the phenomenon of "concentration quenching." Such metal binding agents as 8-hydroxyquinoline and dimethyldithiocarbamic acid, for example, are more active at lower than at higher concentrations in media containing a constant low amount of iron or copper, respectively (13). In contrast, such metal binding agents as the tetracyclines and bacitracin do not become less active as their concentration is increased in an environment with a fixed concentration of metal ions (14). Like the antibiotics, PEBG did not exhibit "concentration quenching"; the drug was completely inactive at levels below  $1 \times 10^{-3}$  M with each test genus. Despite the fact that these results indicate that PEBG does not appear to require an extracellular equimolar concentration of a metallic ion for microstatic activity, it is quite possible that the molecules of the drug that enter the cell can form che-

TABLE I.—EFFECT OF METALLIC IONS ON THE MINIMAL MICROSTATIC CONCENTRATIONS OF PHENETHYL-BIGUANIDE

Genus	Disk	Concentration of PEBG, $\times 10^{-3}$ M <sup>a</sup>							
		0	1	2	3	4	5	10	20
<i>Serratia</i>	Deionized H <sub>2</sub> O	C	C						
	Fe <sup>++</sup> or Fe <sup>+++</sup>	C	C	+	+	+			.
	Other 22 cations	C	C	.					.
<i>Escherichia</i>	Deionized H <sub>2</sub> O	C	C			.			
	Fe <sup>++</sup> or Fe <sup>+++</sup>	C	C	+	+				
	Other 22 cations <sup>a</sup>	C	C						.
<i>Bacillus</i>	Deionized H <sub>2</sub> O	C	C	C	C				
	Fe <sup>++</sup> or Fe <sup>+++</sup>	C	C	C	C	+			
	Other 22 cations	C	C	C	C				
<i>Staphylococcus</i>	Deionized H <sub>2</sub> O	C	C	C					
	Fe <sup>++</sup> or Fe <sup>+++</sup>	C	C	+					
	Other 22 cations	C	C						
<i>Saccharomyces</i>	Deionized H <sub>2</sub> O	C	C						
	Fe <sup>++</sup> or Fe <sup>+++</sup>	C	C	+	+	+			
	Other 22 cations	C	C						
<i>Chlorella</i>	Deionized H <sub>2</sub> O	C	C	C	C	C			
	Fe <sup>++</sup> or Fe <sup>+++</sup>	C	C	C	C	C	+	+	+
	Other 22 cations	C	C	C	C	C			

<sup>a</sup> Complete growth throughout plate C many colonies around disk (no growth in remainder of plate), +, no colonies around disk or in remainder of plate except for aluminum which allowed colonies to appear at a concentration of  $2 \times 10^{-3}$  M

lates with intracellular metal ions and that a 1:1 complex might be the actual toxic entity for specific enzymatic systems

In other experiments, the effect of PEBG on the requirement of zinc for (a) the antibacterial action of bacitracin and (b) the production of prodigiosin by certain strains of *Serratia* was studied and compared with the effect of ethylenediaminetetraacetic acid (EDTA) on these zinc-dependent functions. In contrast to EDTA which can depress the action of bacitracin by depriving the antibiotic of zinc (14), PEBG had no effect on the antibacterial action of the drug (Fig 3). Both EDTA and PEBG prevented the formation of prodigiosin by zinc dependent strains of *Serratia*, but excess zinc was not

able to suppress this action of either of the metal binding agents. In contrast, ferrous iron (which, like zinc, is required for prodigiosin production) was able to reverse the action of both EDTA and PEBG.

Although previous and present chemical studies with biguanides indicate that stable chelates are formed mainly with copper and nickel, the previous and present biological studies indicate that iron-dependent enzymatic systems may be affected by PEBG. The compound has been reported to inhibit a cytochrome oxidase system of rat liver homogenates (15) and to suppress a portion of the electron transport system before cytochrome b of rat liver mitochondria (16). Many portions of this part of the electron transport system are activated by iron (17). This situation may be analogous to that of the tetracyclines which form strong chelates with copper and nickel but which attack iron-dependent enzymes; as with PEBG, iron rather than copper and nickel strongly affects the actions of the tetracyclines (18).

It is apparent from the previous and present biological studies with PEBG that zinc is not involved in the activities of the compound. And since the hypoglycemic action of PEBG is apparently not associated with the utilization of insulin (16), such a result is not unexpected.

In subsequent studies with PEBG it is obvious that the chelating ability of the compound should be considered as both a possible beneficial and a detrimental characteristic of the drug. For example, might side effects of the compound be eliminated by including an iron salt in the PEBG formulation? Or, would a strong chelating agent such as a salt of EDTA potentiate the action of PEBG by reducing the amount of iron available to neutralize the drug? Many additional questions of this nature can be raised, and investigations designed to answer these questions will undoubtedly aid in the more efficient chemotherapeutic usage of the drug as well as in the ultimate elucidation of its biochemical mechanism of action.

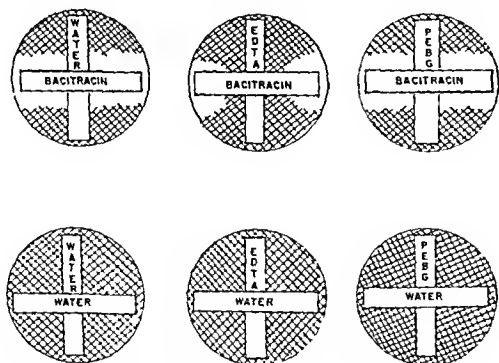


Fig 3.—Extent of visible growth in nutrient agar plates seeded with *Staphylococcus aureus* after two days at 37°. The shaded areas represent areas containing bacterial colonies. The rectangles represent filter paper strips that were briefly soaked in the material stated on the specific rectangle and then placed on the inoculated agar at the beginning of the period of incubation. The concentration of each of the solutions of bacitracin, PEBG, and EDTA was  $1 \times 10^{-3}$  M.

## SUMMARY

1. Evidence is presented for the formation of metal complexes of phenethylbiguanide with copper and nickel.

2. Antimicrobial activities of phenethylbiguanide are not affected by copper or nickel but are depressed by iron.

## REFERENCES

- (1) Chenoweth, M. B., *Pharmacol Revs*, **8**, 57(1956)
- (2) Escovitz, W. E., *Am Rev Tuberc*, **66**, 373(1952)
- (3) Kadota, I., and Tobuyoshi, A., *J Lab Clin Med*, **43**, 375(1954)
- (4) Root, M. A., and Chen, K. K., *J Pharmacol Exptl Therap*, **104**, 404(1952).

- (5) Underwood, E. J., "Trace Elements in Human and Animal Nutrition," Academic Press, Inc., New York, N. Y., 1956, p. 209
- (6) Gilbert, F. A., "Mineral Nutrition and the Balance of Life," University of Oklahoma Press, Norman, Okla., 1957, p. 161
- (7) Shepardson, H. C., *Am Rev Tuberc*, **67**, 544(1953).
- (8) Lepper, M. H., "Aureomycin," Medical Encyclopedia, Inc., New York, N. Y., 1956, p. 33
- (9) Stare, F. J., *Nutrition Revs*, **17**, 81(1959).
- (10) Welcher, F. J., "Organic Analytical Reagents," Vol. 2, D. Van Nostrand Co., Inc., New York, N. Y., 1947, 331
- (11) Ray, P., and Chakravarty, K., *J. Indian Chem. Soc*, **18**, 609(1941)
- (12) Ghosh, S. P., and Banerjee, A. K., *ibid.*, **32**, 32(1955).
- (13) Albert, A., "The Strategy of Chemotherapy," Cambridge University Press, Cambridge, England, 1958, p. 123
- (14) Weinberg, E. D., *Antibiotics Ann*, 1959, 924.
- (15) Steiner, D. F., and Williams, R. H., *Clin Res*, **6**, 55(1958)
- (16) Wick, A. N., Larson, E. R., and Serif, G. S., *J. Biol. Chem*, **233**, 296(1958)
- (17) Nason, A., *Soil Sci*, **85**, 63(1958)
- (18) Weinberg, E. D., *Bact Rev*, **21**, 46(1957).

# The Solubility of Benzoic Acid in Mixed Solvents\*

By MARVIN J. CHERTKOFF† and ALFRED N. MARTIN

The solubility of benzoic acid was determined in mixtures of several solvents of varying polarity. The experimental results were compared with the theoretical solubility as calculated from the Hildebrand equation for regular solutions. The theoretical and experimental results agree well in the region where the solubility parameter of the solute approaches that of the solvent mixture.

MOST LIQUID PHARMACEUTICALS consist of one or more solids dissolved in a mixture of liquids. The solubility of the solids varies with the composition of the solvent mixture, therefore, information which would allow the pharmacist to control the solvent properties of a mixture of liquids would be of great value.

This investigation was undertaken to study the solubility of a solute in a series of mixed solvent systems. Benzoic acid was chosen as a prototype of relatively polar pharmaceutical solids and the solvents were selected to provide a continuous spectra of increasing polarity from hexane to water. The experimental findings were analyzed based on the polarity of the solvent mixture, utilizing the Hildebrand equations for solubility.

A solution which obeys Raoult's law is known as an ideal solution. The solubility of the solute in such a system may be calculated from the heat of fusion of the solute and the heat capacities of this solid and of its supercooled liquid. Hilde-

brand and Scott (1) derived such a relationship,

$$\log x_2 = -\frac{\Delta H_m^F}{4.575} \left( \frac{T_m - T}{TT_m} \right) + \frac{\Delta C_p}{4.575} \left( \frac{T_m - T}{T} \right) - \frac{\Delta C_p}{1.987} \log \left( \frac{T_m}{T} \right) \quad (\text{Eq. 1})$$

where  $x_2$  is the mole fraction solubility at temperature  $T$ ,  $\Delta H_m^F$  the heat of fusion of the solute at its melting point  $T_m$ , and  $\Delta C_p = C_p^l - C_p^s$  where  $C_p^l$  and  $C_p^s$  are the molal heat capacities of the liquid and solid forms, respectively.

For regular solutions (2), those in which heat is absorbed on mixing but no entropy change occurs, short range interactions between the components must be considered. Equation 1 is modified (3) to Eq. 2

$$\log x_2 = -\frac{\Delta H_m^F}{4.575} \left( \frac{T_m - T}{T_m T} \right) + \frac{\Delta C_p}{4.575} \left( \frac{T_m - T}{T} \right) - \frac{\Delta C_p}{1.987} \log \frac{T_m}{T} - \frac{V_2}{4.575 T} (\delta_1 - \delta_2)^2 \phi_1^2 \quad (\text{Eq. 2})$$

in which  $V_2$  is the molar volume of the solute,  $\phi_1$  is the volume fraction of the solvent, and  $\delta_1$  and  $\delta_2$  are the "solubility parameters" of the solvent and solute, respectively. The solubility

\* Received August 21, 1959, from the School of Pharmacy, Purdue University, Lafayette, Ind.

† Supported in part by a grant from the Purdue Research Foundation

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959

† Present address: Merck Sharp & Dohme, Philadelphia 1, Pa.

parameter, as defined by Hildebrand and Scott (4), is the square root of the internal pressure of the substance, a measure of its polarity, so that the more polar a substance the greater its  $\delta$  value. Although Hildebrand restricts the use of his equation to nonpolar solvents, an attempt is made in a later part of this communication to show that the equation for regular solutions is applicable in some systems when the polarity of the solvents are not significantly different from one another.

For two component solvent systems,  $\phi_1$  is taken as the volume fraction of the combined solvents  $a$  and  $b$ , and the new solubility parameters for the mixed solvents may be calculated from

$$\delta_1 = \frac{\phi_a \delta_a + \phi_b \delta_b}{\phi_a + \phi_b} \quad (\text{Eq. 3})$$

where  $\phi_1 = \phi_a + \phi_b$ .

## EXPERIMENTAL

**Reagents.**—Benzoic acid, Fisher certified reagent A. C. S. grade (A-65), recrystallized from chloroform; ethyl acetate, Fisher certified reagent A. C. S. grade (E-145); carbon tetrachloride, spectroanalyzed, Fisher certified reagent A. C. S. grade (C-199); hexane, Viking petroleum solvents, United Fuel Gas Co., Charleston, W. Va., boiling point 65–67°; distilled water, deionized by passage through IRA 120 and IRA 400 resins; commercial absolute ethanol, redistilled and dried over 20-mesh Drierite (5).

**Procedure.**—A slight excess of solid was added to a given volume of the solvent mixture contained in a screw-top bottle. A closure was made with aluminum foil, the top fixed tightly over the foil, and the whole sealed with several turns of electrical tape. The bottles were shaken in a constant temperature bath (Waco Aquarium Vis bath) at  $25 \pm 0.1^\circ$  for twenty-four hours. The equilibrated solutions were then removed, filtered, and samples taken.

The saturated solutions were suitably diluted with 95% ethanol, and the concentration of the benzoic acid was determined spectrophotometrically on a Beckman model DU spectrophotometer at 272.5  $\mu$ .

Densities of the saturated solutions were determined gravimetrically.

## CALCULATIONS

The ideal solubility of benzoic acid was calculated from Eq. 1 using the following data:  $\Delta H_m^\circ = 4302$  cal./mole (6),  $T_m = 395.6^\circ$  (7), and  $\Delta C_p = C_p^l - C_p^s$  (at the melting point) = 13.83 cal./degree/mole (7).

For the calculated solubilities in different solvent mixtures, Eq. 2 was used. The density of the supercooled liquid benzoic acid at  $25^\circ$  was not available in the literature, however the density of the liquid had been reported at several temperatures above its melting point. A plot of density against temperature

Temperature, °C.	Density, Gm./cc.	Reference
122.5	1.080	(8)
125.0	1.077	(9)
130.0	1.078	(8)
130.0	1.075	(10)
131.9	1.074	(11)
155.0	1.052	(10)
180.0	1.029	(10)

gave a linear relationship, and by the method of least squares, the equation for the regression line was found to be

$$d = 1.1926 - (9.064 \times 10^{-4}) t$$

The density at  $25^\circ$  was calculated to be 1.1699 Gm./cc., which gave a molar volume of 104.38 cc./mole.

The solubility parameters used were as follows: Hildebrand and Scott (12) gave  $\delta = 7.3$  for hexane and  $\delta = 8.6$  for carbon tetrachloride. The solubility parameters for *n*-propyl, amyl, *n*-hexyl, *n*-heptyl, and *n*-octyl alcohol were taken from Hildebrand and Scott (13), and those for methyl and *n*-butyl alcohol were calculated from their internal pressures (14). These values were plotted against number of carbon atoms and a smooth curve was fitted. This line crossed the two carbon coordinates at a solubility parameter value of 13.0 which was used for ethyl alcohol.

The solubility parameter of benzoic acid was calculated as 11.5 from the solubility data in hexane. The values for water and ethyl acetate were calculated from the equation (15)

$$\delta = \left( \frac{\Delta H_v^\circ - RT}{V_l} \right)^{1/2} \quad (\text{Eq. 4})$$

and were found to be 23.4 and 9.0, respectively.

In Eq. 4,  $\Delta H_v^\circ$  is the molar heat of vaporization,  $V_l$  is the molar volume at  $25^\circ$ ,  $R$  is the gas constant, and  $T$  is the absolute temperature. For water, the heat of vaporization at  $25^\circ$  was taken as 582.2 cal./Gm., an average of the values at  $20^\circ$  and  $30^\circ$  (16).

For ethyl acetate, the following heats of vaporization were plotted and the value at  $25^\circ$  read as 97.5 cal./Gm.:

Temperature, °C.	$\Delta H_v^\circ$ , cal./Gm.	Reference
0.0	102.00	(17)
76.0	87.63	(18)
76.5	87.97	(19)

An example of these calculations for the solubility of benzoic acid in a mixture of equal volumes of ethyl acetate and hexane at  $25^\circ$  follows:  
From Eq. 3

$$\delta_1 = \frac{\phi_a \delta_a + \phi_b \delta_b}{\phi_a + \phi_b} = \frac{(0.5 \times 7.3) + (0.5 \times 9.0)}{0.5 + 0.5} = 8.15$$

Then employing Eq. 2

$$\log x_2 = - \frac{4302 (395.6 - 298.2)}{4.575 \times 395.6 \times 298.2} + \frac{13.83 (395.6 - 298.2)}{4.575 \times 298.2} - \frac{13.83}{1.987} \log \frac{395.6}{298.2} - \frac{104.38 (11.5 - 8.15)^2 (1)^2}{4.575 \times 298.2}$$

$$\log x_2 = 1.5014 \quad x_2 = 0.03152$$

In the above calculation,  $\phi_1$  was taken as equal to one. In more concentrated solutions this introduces a substantial error, so that the method of successive approximations was applied to obtain a theoretical  $\phi_1$ . Using Eq. 5, which was obtained by combining

$$\phi_1 = \frac{(1 - x_2) V_1}{(1 - x_2) V_1 + x_2 V_2} \quad (\text{Eq. 5})$$

the equations for volume fraction and mole fraction, and the value for  $x_2$  obtained above, a new  $\phi_1$  was calculated which was then used in Eq. 2 to recalculate  $x_2$ . This procedure was repeated several times until  $x_2$  did not change significantly and a final theoretical mole fraction solubility was obtained. For practical work these values would, of course, be converted to the molal scale, or knowing the density of the solution, to molarity.

The values for  $V_1$ , the molar volume of the solvent, were calculated as weighted averages of the molar volume of the components of the solvent mixture in a manner similar to that used for the calculation of  $\delta$  values of the mixed solvents. The error introduced by the assumption that molar volumes are additive was accepted to simplify the calculations.

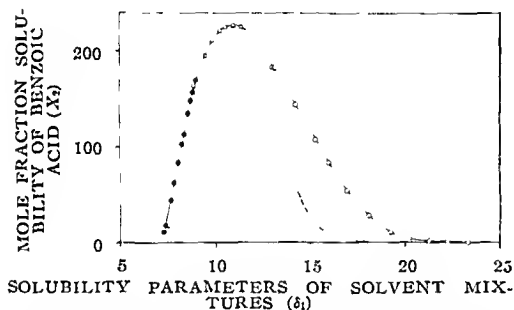


Fig. 1.—Mole fraction solubility of benzoic acid vs. solubility parameters of the solvent mixtures hexane-ethyl acetate, ethyl acetate-ethyl alcohol, ethyl alcohol-water. — observed, ● hexane-ethyl acetate, □ ethyl acetate-ethyl alcohol, ○ ethyl alcohol-water; --- calculated.

## DISCUSSION

From Eq. 2 it is obvious that when  $\delta_1 = \delta_2$ , the last term becomes equal to zero, Eq. 2 becomes identical with Eq. 1, and the ideal solubility of the solute should be observed. If a pair of miscible liquids are chosen so that their solubility parameters lie on either side of that for a solute, the volume fractions of the two which will make  $\delta_1 = \delta_2$  can be calculated from Eq. 3. Such a mixture would be an "ideal" solvent for that solute; that is, the solute would exhibit the solubility predicted by the ideal solubility equation, provided the solution meets the criteria of a regular solution as defined by Hildebrand and Scott. Also, since the last term of Eq. 2 is negative, its presence will lower the value of  $x_2$ ; when this last term is equal to zero, the maximum solubility should be observed.

Figure 1 shows the results of the calculated and observed mole fraction solubilities of benzoic acid ( $x_2$ ) versus the solubility parameters of the solvent mix-

tures ethyl acetate-hexane, ethyl acetate-ethanol, and ethanol-water. The calculated curve reached a maximum peak at  $\delta = 11.5$ , since this was the solubility parameter for benzoic acid. The experimental curve exhibited a plateau region from about 10.2 to 11.8.

The similarity of these two curves is surprising, since such excellent agreement is not often observed in solubility work; furthermore, the high polarity and hydrogen bonding in the system makes these results all the more interesting. The calculated ideal solubility of benzoic acid at  $\delta_1 = 11.5$  ( $x_2 = 0.2275$ ) lies on the experimental curve. Gordon and Scott (20) investigated a nonpolar system, phenanthrene dissolved in a mixture of methylene iodide and cyclohexane having various  $\delta$  values, and found that while the maximum solubility occurred where  $\delta_{\text{solvent}} \cong \delta_{\text{solute}}$ , the experimental solubility was considerably below that of the ideal value.

In the present study, good agreement was found between observed and calculated solubility from about 50 to 100% v/v ethyl alcohol in ethyl acetate ( $\delta = 11.0$  to 13.0). As the concentration of ethyl acetate increased, the theoretical and experimental values no longer coincided, as observed in the left hand portion of the curve in Fig. 1. The same behavior was observed as water was added, that is, for  $\delta$  values of 14.0 to 23.4. Interactions between the solute and these solvents would cause a change in entropy, creating a nonregular solution, and thus the Hildebrand treatment would not be expected to apply. In view of this, the surprising fact is not that deviation occurred over most of the curves, but that the curves coincided in the region of the maximum ( $\delta = 11.5$ ). Perhaps the magnitude of the interactions in the peak area were such that deviations from regular solution theory nullified one another.

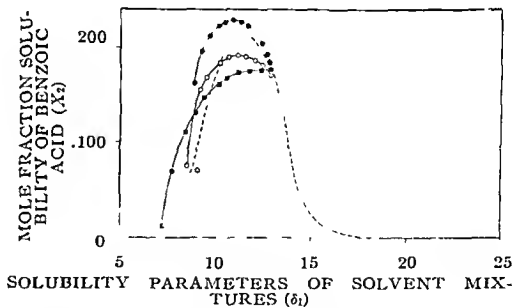


Fig. 2.—Mole fraction solubility of benzoic acid vs. solubility parameters of the solvent mixtures hexane-ethyl alcohol, carbon tetrachloride-ethyl alcohol, ethyl acetate-ethyl alcohol. — observed, ■ hexane-ethyl alcohol, ○ carbon tetrachloride-ethyl alcohol, ● ethyl acetate-ethyl alcohol; --- calculated.

Additional experimentation was conducted to verify these findings. Systems were chosen so that the  $\delta$  values of the two liquids in a given solvent mixture bracketed the  $\delta$  value of benzoic acid. Figure 2 demonstrates the solubility obtained for benzoic acid in three mixed solvent systems. In each case, ethanol was taken as the solvent with the  $\delta$  value (13.0)

higher than that of benzoic acid (11.5) since it was completely miscible with all the other liquids used in this study. As the solubility parameters of the low polarity solvents, hexane (7.3), carbon tetrachloride (8.6), and ethyl acetate (9.0) approached that of ethanol (13.0), the curve of experimentally determined solubility approached the theoretical curve. In the hexane-ethanol system, neither qualitative nor quantitative agreement of experimental and theoretical results was observed. Qualitative agreement was found in the carbon tetrachloride-ethanol system, that is, the maximum solubility was observed at approximately the theoretically predicted value, however, quantitative agreement was not good.

In the ethyl acetate-ethanol system both qualitative and quantitative correlations were obtained. From a practical standpoint, these results suggested that in order to attain the maximum solubility of a compound in a mixed solvent system, the solvents should not only provide a  $\delta$  value equal to that of the solute, but furthermore, the individual solvents of the mixture should not be too widely separated in polarity. Further investigations on the application of the Hildebrand formulation to solutions of moderately high polarity must be conducted to determine if the correspondence observed in these systems occurs with other polar solvents and solutes.

## REFERENCES

- (1) Hildebrand, J. H., and Scott, R. L., "The Solubility of Nonelectrolytes," 3rd ed., American Chemical Society Monograph No. 17, Reinhold Publishing Corp., New York, N. Y., 1950, p. 27.
- (2) *Ibid.*, p. 122.
- (3) *Ibid.*, p. 271.
- (4) *Ibid.*, p. 129.
- (5) Weissberger, A., Proskauer, E. S., Riddick, J. A., and Toops, E. E., "Techniques of Organic Chemistry," Vol. VII, "Organic Solvents," Interscience Publishers Inc., New York, N. Y., 1955, p. 340.
- (6) Furukawa, G. T., McCoskey, R. E., and King, G. J., *J. Research Natl. Bur. Standards*, **47**, 256 (1951).
- (7) Ginnings, D. C., and Furukawa, G. T., *J. Am. Chem. Soc.*, **75**, 522 (1953).
- (8) Dunstan, A. E., Hilditch, T. P., and Thole, F. B., *J. Chem. Soc.*, 103, 133 (1913).
- (9) Baskow, A., *J. Russ. Phys. Chem. Soc.*, **50**, 614 (1918); Through Beilstein's "Handbuch der Organischen Chemie," Erstes Ergänzungswork, Band 1X, Julius Springer, Berlin, 1932, p. 55.
- (10) Burriel Marti, F., *Bull. soc. chim. Belges*, **39**, 590 (1930).
- (11) Eijkman, M. J. F., *Rec. trav. chim.*, **12**, 184 (1893).
- (12) Hildebrand, J. H., and Scott, R. L., "The Solubility of Nonelectrolytes," 3rd ed., American Chemical Society Monograph No. 17, Reinhold Publishing Corp., New York, N. Y., 1950, pp. 435, 436.
- (13) *Ibid.*, p. 388.
- (14) *Ibid.*, p. 283.
- (15) *Ibid.*, p. 424.
- (16) Hodgman, C. P., "Handbook of Chemistry and Physics," 34th ed., Chemical Rubber Co., Cleveland, Ohio, 1952, p. 1973.
- (17) *Ibid.*, p. 1975.
- (18) Mathews, J. H., *J. Am. Chem. Soc.*, **48**, 562 (1926).
- (19) Tyrer, D., *J. Chem. Soc.*, 101, 81 (1912).
- (20) Gordon, L. J., and Scott, R. L., *J. Am. Chem. Soc.*, **74**, 4138 (1952).

## The Photo-oxidation of Dihydroxyphenylalanine in the Presence of 8-Methoxypsoralen\*

By JOSEPH JUDIS

8-Methoxypsoralen (8-MOP) is well known for its ability to stimulate melanin formation in vitiligo or normal skin if administration is coupled with exposure to sunlight or ultraviolet light. As an approach to studying the mechanism of action of 8-MOP, the latter was incubated with DOPA and exposed to various types of light. Sunlight, white light, or long wave ultraviolet light (3660 Å.) stimulated the oxidation of DOPA in the presence of 8-MOP but not in the absence of the latter. 8-MOP protected DOPA against short wavelength ultraviolet (2540 Å.) although DOPA was oxidized by the latter in the absence of 8-MOP. The photo-oxidation of DOPA in the presence of long wavelength ultraviolet light and 8-MOP was more rapid at pH values above 7 and was completely inhibited by ascorbic acid, cysteine, cysteamine,  $\beta$ -aminoethylisothiuronium bromide HBr, and partially inhibited by sodium thioglycollate. The possible implication of these *in vitro* observations to the mode of action of 8-MOP *in vivo* is discussed.

**A** CRYSTALLINE PRINCIPLE, 8-methoxypsoralen (8-MOP),<sup>1</sup> found in the fruit of the plant *Ammi majus* Linn., is one of the several furocoumarins recently isolated from this source (1). At the present time, 8-MOP is widely used, both orally and topically, as a treatment for vitiligo

(2, 3) as well as for the promotion of suntanning in normal persons (4, 5, 6).

While it has been clearly established that 8-MOP stimulates pigmentation of the skin, its mechanism of action is not understood. Pigmentation is due to the formation of melanin by a number of steps (7-10) beginning with tyrosinase. The classic scheme (10) for the enzymatic oxidation of tyrosine consists of the following: (a) oxidation in the presence

\* Received August 21, 1959, from the College of Pharmacy, University of Toledo, Toledo, Ohio.  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

<sup>1</sup> Hereafter, 8-methoxypsoralen will be abbreviated as 8-MOP, and dihydroxyphenylalanine will be abbreviated as DOPA.

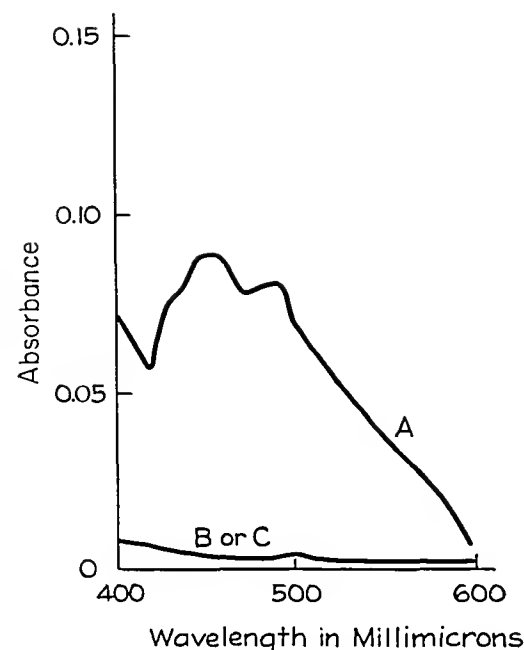


Fig. 1.—Photo-oxidation of DOPA by 8-MOP in white light. A, DOPA plus 8-MOP in white light; B, DOPA without 8-MOP in white light; C, DOPA plus 8-MOP in the dark. Time of irradiation: forty-five minutes.

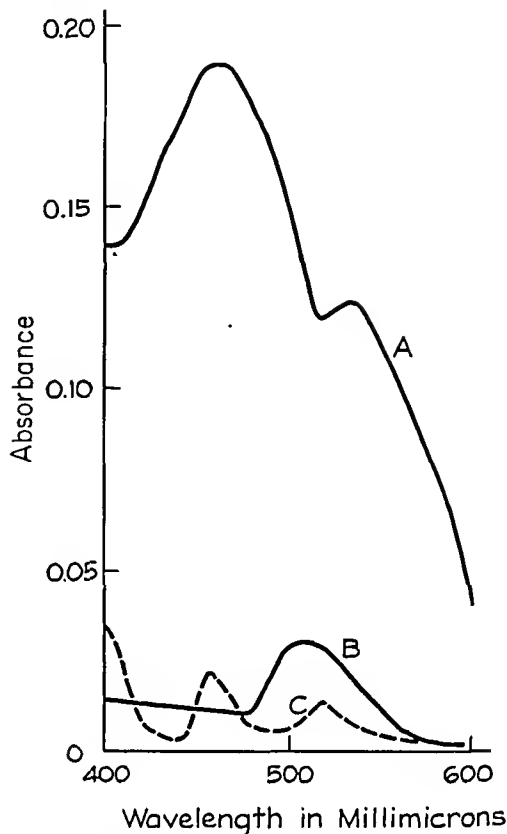


Fig. 2.—Photo-oxidation of DOPA by 8-MOP in "black light." A, DOPA plus 8-MOP in "black light;" B, DOPA without 8-MOP in "black light;" C, DOPA plus 8-MOP in the dark. Time of irradiation: Thirty-five minutes.

DOPA; (b) further oxidation of DOPA by tyrosinase to DOPA quinone; (c) condensation to a leuco compound; (d) oxidation to DOPA chrome; (e) decarboxylation and reduction to 5,6-dihydroxyindole; (f) oxidation to indole 5,6-quinone; (g) polymerization and oxidation to DOPA melanin. *In vivo*, the quinones would react readily, before or after polymerization, with the SH and  $\text{NH}_2$  groups of proteins to form a melanoprotein compound.

It is well known that the sequence of events leading to melanin formation is influenced by a number of factors such as copper, an essential part of tyrosinase; sulfhydryl groups, which inhibit and thus regulate the tyrosine-tyrosinase reaction; various hormones such as the melanocyte-stimulating hormone (MSH), thyroid, adrenal, and gonadal; temperature; electrolyte concentration; pH and oxidation-reduction potential. Since the stimulatory effect of 8-MOP on melanin formation is clearly a photochemical one, it seemed of interest to see whether a key compound, such as tyrosine or DOPA could be photochemically oxidized to melanin, using 8-MOP as the photosensitizer. In preliminary experiments, tyrosine was not photo-oxidized under the conditions used but DOPA was, so the photo-oxidation of the latter compound was studied.

## MATERIALS AND METHODS

**Materials.**—DOPA was obtained from the California Corp. for Biochemical Research or Nutritional Biochemicals Corp. The ascorbic acid, cysteine hydrochloride, and cysteamine hydrochloride were obtained from the California Corp. for Biochemical Research. The disodium versenate was supplied by Dow Chemical Corp., the sodium thioglycollate was obtained from Baltimore Biological Laboratories, and the AET ( $\beta$ -aminoethylisothiuronium bromide hydrobromide) was purchased from Schwartz Laboratories. The 8-MOP was used as the 1% lotion in isopropanol, acetone, and alcohol marketed by the Paul B. Elder Co., Bryan, Ohio, under the trademark of Oxsoralen. The other psoralen derivatives were a gift of the Paul B. Elder Co. The phosphate buffers of various pH's were prepared by mixing the proper proportions of 0.067  $M$   $\text{KH}_2\text{PO}_4$  and 0.067  $M$   $\text{Na}_2\text{HPO}_4$ .

The artificial light sources were as follows: (a) white light, two 150-watt Sylvania flood lamps; (b) long wavelength ultraviolet, G. E. 15-watt "black light" emitting most of its radiations at approximately 3660 Å.; (c) short wavelength ultraviolet, G. E. 15-watt germicidal lamp emitting most of its radiation at 2540 Å.

**Procedure.**—The typical experimental procedure was as follows: the reaction mixtures were placed in 16 × 150 mm. Kimax screw-cap test tubes at a distance of 10 cm. from the light source in the case of irradiation with "black light" or white light. Absorbance readings were made on the Spectronic 20 spectrophotometer. Absorption spectra were usually performed on the Beckman DU quartz spectrophotometer using the appropriate 1-cm. silica cells. When the G. E. germicidal lamp was used instead of the "black light," quartz test tubes, approximately 15 × 150 mm, were substituted for the Kimax test tubes. In irradiating with white light, the test tubes were kept 18 cm. from the light source to allow for a water filter for absorption of the heat produced.

The reaction mixtures normally contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, and enough 0.067 *M* phosphate buffer of the appropriate pH to make a total of 6.0 ml. If other ingredients were in the reaction mixture, an equivalent volume of the buffer was omitted.

## RESULTS AND DISCUSSION

Lerner and his co-workers (11) incubated ultraviolet irradiated 8-MOP with tyrosinase and either tyrosine or DOPA. They found no effects on oxygen uptake or pigment formation. In the present study, DOPA and 8-MOP were incubated together and irradiated. Figure 1 gives the absorption spectrum curve of a reaction mixture irradiated with white light. The amount of pigment formed is not nearly

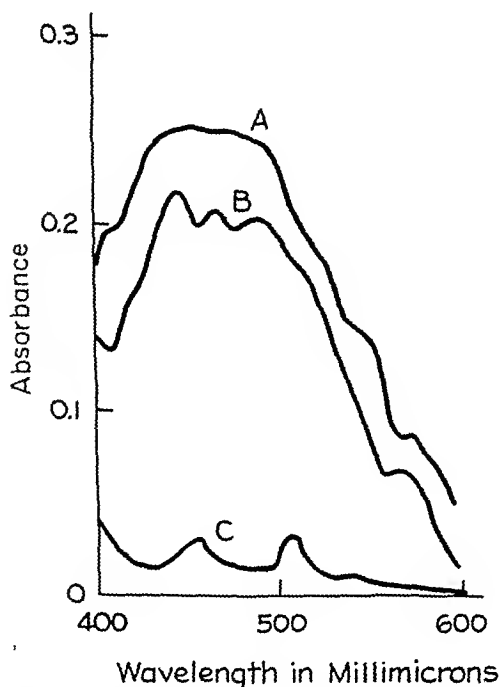


Fig. 3.—Photo-oxidation of DOPA in ultraviolet light. A, DOPA without 8-MOP in ultraviolet light; B, DOPA plus 8-MOP in ultraviolet light; C, DOPA plus 8-MOP in the dark. Time of irradiation: Thirty-five minutes.

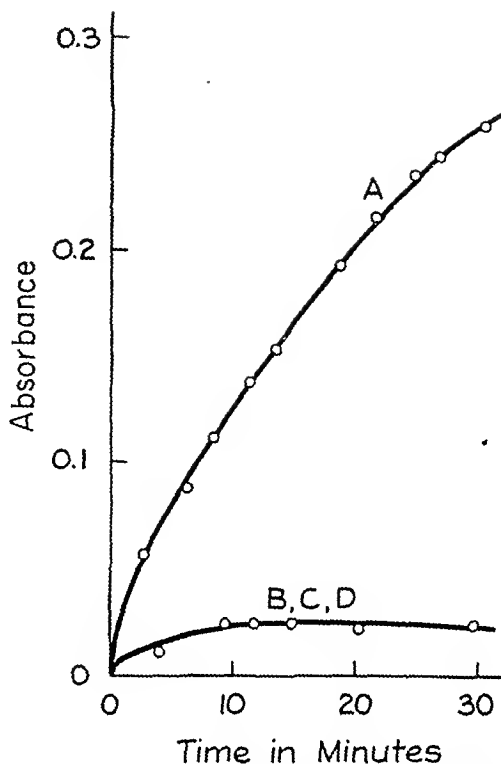


Fig. 4.—Photo-oxidation of DOPA by 8-MOP in "black light." A, DOPA plus 8-MOP in "black light;" B, DOPA without 8-MOP in "black light;" C, DOPA plus 8-MOP in the dark; D, 8-MOP in "black light." Absorbance was measured at 480  $m\mu$ .

so great as when "black light" is used as the source of light energy (see Fig. 2). With short wavelength ultraviolet light, the absorption curves (Fig. 3) of the reaction mixtures seem to indicate that the 8-MOP protects the DOPA against the ultraviolet light. This is a reasonable explanation since it is known from the ultraviolet absorption curve of 8-MOP (12) that the latter does absorb ultraviolet light at approximately 250  $m\mu$  as well as at approximately 300  $m\mu$ .

Photo-oxidation of DOPA proceeded rather rapidly with long wavelength ultraviolet as the source of radiant energy. A typical kinetic picture is shown in Fig. 4. A similar kinetic picture was obtained with two other psoralen derivatives, 8-isomethylpsoralen and 2',8-dimethylpsoralen.

The effect of pH on the photo-oxidative reaction was found to be similar to that obtained by Hirsch (13) who studied DOPA auto-oxidation as a function of pH. He found melanin yields from DOPA in a given time period to increase as the pH increased. The effect of pH on the photo-oxidation of DOPA is shown in Fig. 5.

There has been a great deal of study on the ability of certain pure chemicals and tissue and blood constituents to inhibit the auto-oxidation of DOPA (14-17). Various workers feel that the body has a chemical mechanism for regulating DOPA oxidation as well as the oxidation of other physiological phenols. This mechanism appears to involve —SH



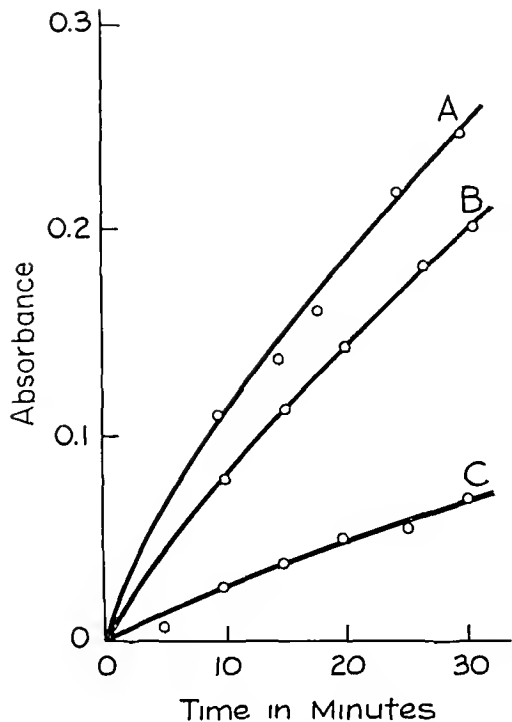


Fig 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light " A, pH 8.05, B, pH 6.6, C, pH 5.3 Absorbance was measured at 480 mμ.

groups Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation A couple of compounds believed to be radioprotective by a chelating mechanism were also included. Table I gives the results obtained

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22) Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8) The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit <sup>a</sup>
KCN	0.03 M	—
Disodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>b</sup>	0.01 M	+
Cysteamine HCl	0.01 M	+
AET	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg of 8 MOP, 1 mg of DOPA, 1 ml of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 M phosphate buffer, and enough phosphate buffer to make 6.0 ml

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24). In this disease, it is possible that 8-MOP may act in several ways: (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation All of these speculations remain to be proved.

## REFERENCES

- (1) Fitzpatrick, T. B., and Pathak, M. A., *J. Invest Dermatol.*, **32**, 229 (1959)
- (2) London, I. D., *ibid.*, **32**, 315 (1959)
- (3) Jarrett, A., and Szabo, G., *Brit. J. Dermatol.*, **68**, 313 (1956)
- (4) Stegmaier, O. C., *J. Invest Dermatol.*, **32**, 345 (1959)
- (5) Daniels, F., Jr., Hopkins, C. E., Imbrie, J. D., Bergeron, L., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, **32**, 321 (1959)
- (6) Kanof, N. B., *ibid.*, **24**, 5 (1955)
- (7) Lerner, A. B., *Am. J. Med.*, **19**, 902 (1955)
- (8) Levin, H. M., *Quart. Bull. Northwestern Univ. Med. School*, **30**, 1 (1956)
- (9) Fitzpatrick, T. B., and Szabo, G., *J. Invest Dermatol.*, **32**, 197 (1959)
- (10) Lerner, A. B., and Case, J. D., *ibid.*, **32**, 211 (1959)
- (11) Lerner, A. B., Denton, C. R., and Fitzpatrick, T. B., *ibid.*, **20**, 299 (1953)
- (12) Fowlks, W. L., *ibid.*, **32**, 249 (1959)
- (13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330
- (14) Hirsch, H. M., *Cancer Research*, **16**, 1076 (1956)
- (15) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *Arch. Biochem. Biophys.*, **72**, 255 (1957)
- (16) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *ibid.*, **72**, 271 (1957)
- (17) Monder, C., Williams, J. N., Jr., and Waisman, H. A., *ibid.*, **75**, 46 (1958)
- (18) Hirsch, H. M., *Radiation Research*, **5**, 9 (1956)
- (19) Isaka, S., and Akino, M., *Nature*, **177**, 184 (1956)
- (20) Isaka, S., *ibid.*, **179**, 578 (1957)
- (21) Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H., *J. Biol. Chem.*, **187**, 793 (1950)
- (22) Daniels, F., Jr., *J. Invest Dermatol.*, **32**, 147 (1959)
- (23) Pinkus, H., *ibid.*, **32**, 281 (1959)
- (24) Lerner, A. B., *ibid.*, **32**, 285 (1959)

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit
KCN	0.03 M	—
Disodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>b</sup>	0.01 M	+
Cysteamine HCl	0.01 M	+
AET	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, an appropriate amount of the substance dissolved in pH 6.3, 0.067 M phosphate buffer, and enough phosphate buffer to make 6.0 ml.

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome. This is presumably due to the photo-oxidation of sodium thioglycollate. In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24). In this disease, it is possible that 8-MOP may act in several ways: (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation. All of these speculations remain to be proved.

# REFERENCES

- (1) Fitzpatrick, T. B., and Pathak, M. A., *J. Invest. Dermatol.*, 32, 229 (1959)
- (2) London, I. D., *ibid.*, 32, 315 (1959)
- (3) Stegmaier, O. C., and Szabo, G., *Brit. J. Dermatol.*, 68, 313 (1956)
- (4) Daniels, R., Jr., Hopkins, C. B., Imbrie, J. D., Bergeron, T., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, 32, 321 (1959)
- (5) Knaul, N. B., *ibid.*, 24, 5 (1955)
- (6) Lerner, A. B., *ibid.*, 19, 902 (1955)
- (7) Levin, H. M., *Quart. Bull. Northwestern Univ. Med. School*, 30, (1956)
- (8) Lerner, T. B., and Szabo, G., *J. Invest. Dermatol.*, 32, 197 (1959)
- (9) Lerner, A. B., and Case, J. D., *ibid.*, 32, 211 (1959)
- (10) Fowles, W. L., *ibid.*, 32, 249 (1959)
- (11) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y. 1959, p. 330
- (12) Hirsch, H. M., *Cancer Research*, 16, 1076 (1956)
- (13) Mondler, C., Weisman, H. A., and Williams, J. N., *Arch. Biochem. Biophys.*, 72, 255 (1957)
- (14) Mondler, C., Weisman, H. A., and Williams, J. N., *ibid.*, 72, 271 (1957)
- (15) Mondler, C., Williams, J. N., Jr., and Weisman, H. A., *ibid.*, 75, 46 (1958)
- (16) Hirsch, H. M., *Radiation Research*, 5, 91 (1956)
- (17) Isaka, S., and Akino, M., *Nature*, 177, 184 (1956)
- (18) Lerner, A. B., Fitzpatrick, T. B., and Caikins, E., and Summerson, W. H., *J. Biol. Chem.*, 187, 793 (1950)
- (19) Daniels, R., Jr., *J. Invest. Dermatol.*, 32, 147 (1959)
- (20) Pincus, H., *ibid.*, 32, 281 (1959)
- (21) Lerner, A. B., *ibid.*, 32, 283 (1959)

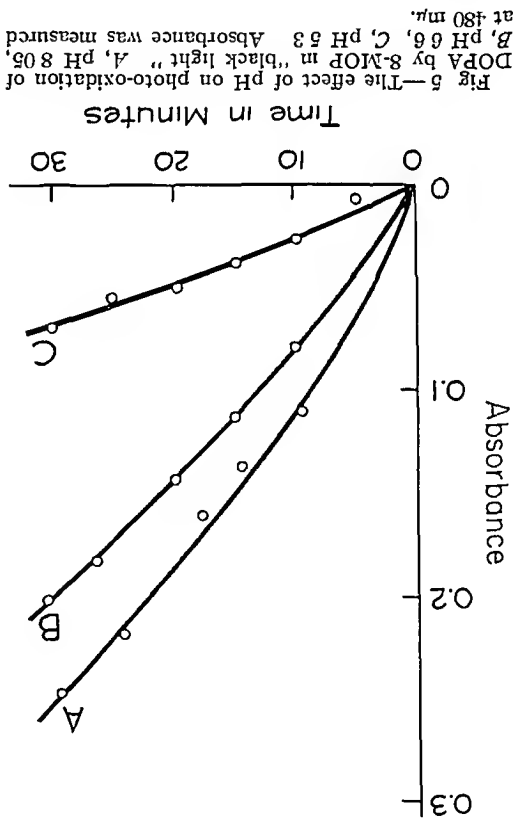


Fig. 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light": A, pH 8.05; B, pH 6.6; C, pH 5.3. Absorbance was measured at 480 mμ.

Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation. Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation. While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals. It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation. A couple of compounds believed to be radioprotective by a chelating mechanism were also included. Table I gives the results obtained. It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22). Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known. Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8). The suntanning process could possibly be due to its mechanism by which 8-MOP may stimulate the

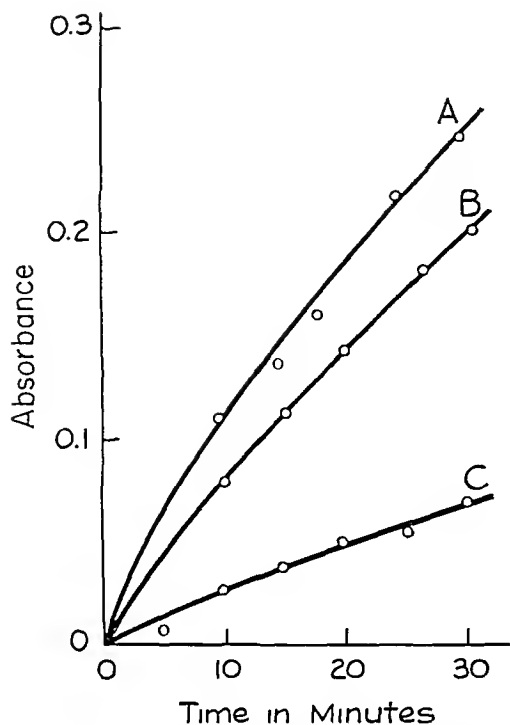


Fig 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light" A, pH 8.05, B, pH 6.6, C, pH 5.3 Absorbance was measured at 480 mμ.

groups Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation A couple of compounds believed to be radioprotective by a chelating mechanism were also included Table I gives the results obtained

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22). Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8). The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit <sup>a</sup>
KCN	0.03 M	—
Disodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>b</sup>	0.01 M	+
Cysteamine HCl	0.01 M	+
AET	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 M phosphate buffer and enough phosphate buffer to make 6.0 ml.

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24) In this disease, it is possible that 8-MOP may act in several ways: (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation All of these speculations remain to be proved.

## REFERENCES

- (1) Fitzpatrick, T. B., and Pathak, M. A., *J. Invest Dermatol*, **32**, 229(1959)
- (2) London, I. D., *ibid.*, **32**, 315(1959)
- (3) Jarrett, A., and Szabo, G., *Brit J Dermatol*, **68**, 313(1956)
- (4) Stegmaier, O. C., *J. Invest Dermatol*, **32**, 345(1959).
- (5) Daniels, F., Jr., Hopkins, C. E., Imbrie, J. D., Bergeron, L., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, **32**, 321(1959).
- (6) Kanof, N. B., *ibid.*, **24**, 5(1955)
- (7) Lerner, A. B., *Am. J. Med.*, **19**, 902(1955)
- (8) Levin, H. M., *Quart. Bull. Northwestern Univ. Med. School*, **30**, 1(1956)
- (9) Fitzpatrick, T. B., and Szabo, G., *J. Invest Dermatol*, **32**, 197(1959)
- (10) Lerner, A. B., and Case, J. D., *ibid.*, **32**, 211(1959)
- (11) Lerner, A. B., Denton, C. R., and Fitzpatrick, T. B., *ibid.*, **20**, 299(1953).
- (12) Fowles, W. L., *ibid.*, **32**, 249(1959).
- (13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330
- (14) Hirsch, H. M., *Cancer Research*, **16**, 1076(1956)
- (15) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *Arch. Biochem. Biophys.*, **72**, 255(1957).
- (16) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *ibid.*, **72**, 271(1957)
- (17) Monder, C., Williams, J. N., Jr., and Waisman, H. A., *ibid.*, **75**, 46(1958).
- (18) Hirsch, H. M., *Radiation Research*, **5**, 9(1956)
- (19) Isaka, S., and Akino, M., *Nature*, **177**, 184(1956).
- (20) Isaka, S., *ibid.*, **179**, 578(1957)
- (21) Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H., *J. Biol. Chem.*, **187**, 793(1950)
- (22) Daniels, F., Jr., *J. Invest Dermatol*, **32**, 147(1959).
- (23) Pinkus, H., *ibid.*, **32**, 281(1959)
- (24) Lerner, A. B., *ibid.*, **32**, 285(1959).

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit
KCN	0.03 M	—
Disodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>b</sup>	0.01 M	+
Cysteamine HCl	0.01 M	+
ABT	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 M phosphate buffer, and enough phosphate buffer to make 6.0 ml.

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome.

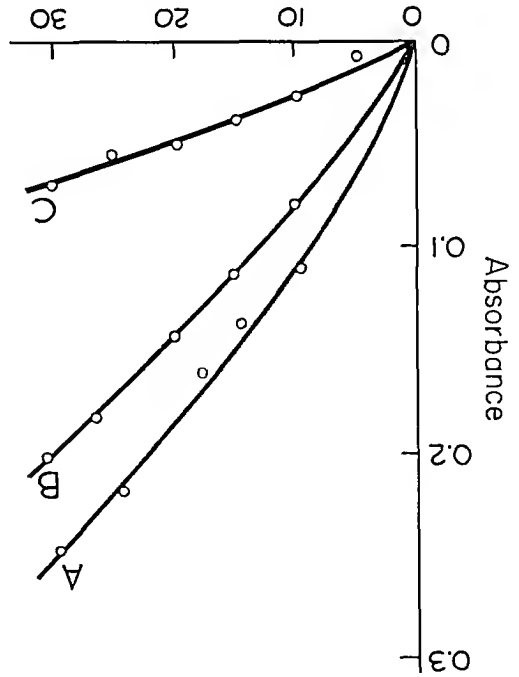
accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome. This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24). In this disease, it is possible that 8-MOP may act in several ways: (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation. All of these speculations remain to be proved.

## REFERENCES

- (1) Fitzpatrick, J. B., and Pathak, M. A., *J. Invest. Dermatol.*, **32**, 229 (1959).
- (2) London, I. D., *ibid.*, **32**, 315 (1959).
- (3) Jarratt, J. C., and Szabo, C., *Brit. J. Dermatol.*, **68**, 313 (1956).
- (4) Stegmaier, O. C., *J. Invest. Dermatol.*, **32**, 345 (1959).
- (5) Daniels, F., Jr., Hopkins, C. B., Imbrie, J. D., Berkson, I., Crowe, F., and Fitzpatrick, J. B., *ibid.*, **32**, 321 (1959).
- (6) Kanof, N. B., *ibid.*, **24**, 5 (1955).
- (7) Lerner, A. B., *Ann. N.Y. Acad. Sci.*, **19**, 902 (1955).
- (8) Lerner, H. M., *Quart. Bull. Northwestern Univ. Med. School*, **30**, 1 (1956).
- (9) Fitzpatrick, J. B., and Szabo, C., *J. Invest. Dermatol.*, **32**, 209 (1959).
- (10) Lerner, A. B., and Case, J. D., *ibid.*, **32**, 211 (1959).
- (11) Lerner, A. B., Denon, C. R., and Fitzpatrick, J. B., *ibid.*, **20**, 209 (1953).
- (12) Rowles, W. L., *ibid.*, **32**, 249 (1959).
- (13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330.
- (14) Hirsch, H. M., *Cancer Research*, **16**, 1076 (1956).
- (15) Lerner, A. B., and Williams, J. N., *Jr. Arch. Biochem. Biophys.*, **72**, 235 (1957).
- (16) Alexander, C., Waisman, H. A., and Williams, J. N., *Jr. Arch. Biochem. Biophys.*, **72**, 235 (1957).
- (17) Alexander, C., Williams, J. N., Jr., and Waisman, H. A., *ibid.*, **75**, 46 (1958).
- (18) Hirsch, H. M., *Radiation Research*, **5**, 9 (1956).
- (19) Isaka, S., and Alamo, M., *Nature*, **177**, 181 (1956).
- (20) Isaka, S., *ibid.*, **179**, 578 (1957).
- (21) Lerner, A. B., Fitzpatrick, J. B., Calkins, E., and Summerson, W. H., *J. Biol. Chem.*, **187**, 793 (1950).
- (22) Daniels, F., Jr., *J. Invest. Dermatol.*, **32**, 117 (1959).
- (23) Pinkus, H., *ibid.*, **32**, 281 (1959).
- (24) Lerner, A. B., *ibid.*, **32**, 285 (1959).

Fig. 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light": (a) pH 8.05, B, pH 6.6, C, pH 5.3. Absorbance was measured at 480 mμ.



Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation. Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation. While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals. It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation. A couple of compounds believed to be radioprotective by a chelating mechanism were also included. Table I gives the results obtained.

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22). Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known. Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8). The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

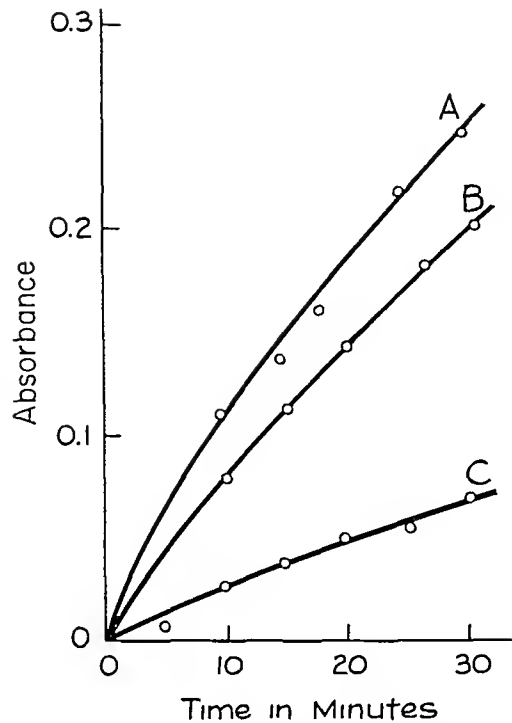


Fig 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light" A, pH 8.05, B, pH 6.6, C, pH 5.3 Absorbance was measured at 480 mμ.

groups Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation-induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation. A couple of compounds believed to be radioprotective by a chelating mechanism were also included Table I gives the results obtained

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22) Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8) The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit <sup>a</sup>
KCN	0 03 M	—
Sodium versenate	0 02 M	—
Ascorbic acid	0 002 M	+
Cysteine HCl	0 005 M	+
Sodium thioglycollate <sup>b</sup>	0 01 M	+
Cysteamine HCl	0 01 M	+
AET	0 01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg of 8-MOP, 1 mg of DOPA, 1 ml of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 M phosphate buffer, and enough phosphate buffer to make 6.0 ml

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24). In this disease, it is possible that 8-MOP may act in several ways: (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation All of these speculations remain to be proved.

## REFERENCES

- (1) Fitzpatrick, T. B., and Pathnk, M. A., *J. Invest Dermatol*, 32, 229(1959)
- (2) London, I. D., *ibid.*, 32, 315(1959)
- (3) Jarrett, A., and Szabo, G., *Brit. J. Dermatol*, 68, 313(1956)
- (4) Stegmaier, O. C., *J. Invest Dermatol*, 32, 345(1959)
- (5) Daniels, F., Jr., Hopkins, C. E., Imbrie, J. D., Bergeron, L., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, 32, 321(1959)
- (6) Kanof, N. B., *ibid.*, 24, 5(1955)
- (7) Lerner, A. B., *Am. J. Med.*, 19, 902(1955)
- (8) Levin, H. M., *Quart. Bull. Northwestern Univ. Med School*, 30, 1(1956)
- (9) Fitzpatrick, T. B., and Szabo, G., *J. Invest Dermatol*, 32, 197(1959)
- (10) Lerner, A. B., and Case, J. D., *ibid.*, 32, 211(1959)
- (11) Lerner, A. B., Denton, C. R., and Fitzpatrick, T. B., *ibid.*, 20, 299(1953)
- (12) Fowlks, W. L., *ibid.*, 32, 249(1959)
- (13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330
- (14) Hirsch, H. M., *Cancer Research*, 16, 1076(1956)
- (15) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *Arch. Biochem. Biophys.*, 72, 255(1957)
- (16) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *ibid.*, 72, 271(1957)
- (17) Monder, C., Williams, J. N., Jr., and Waisman, H. A., *ibid.*, 75, 46(1958)
- (18) Hirsch, H. M., *Radiation Research*, 5, 9(1956)
- (19) Isaka, S., and Akino, M., *Nature*, 177, 184(1956)
- (20) Isaka, S., *ibid.*, 179, 578(1957)
- (21) Lerner, A. B., Fitzpatrick, T. B., Callins, E., and Summerson, W. H., *J. Biol. Chem.*, 187, 793(1950)
- (22) Daniels, T., Jr., *J. Invest Dermatol*, 32, 147(1959)
- (23) Pinkus, H., *ibid.*, 32, 281(1959)
- (24) Lerner, A. B., *ibid.*, 32, 285(1959)

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit
KCN	0.03 M	—
Sodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>a</sup>	0.01 M	+
Cysteine HCl	0.01 M	+
ABT	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 M phosphate buffer, and enough phosphate buffer to make 6.0 ml.  
<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome.

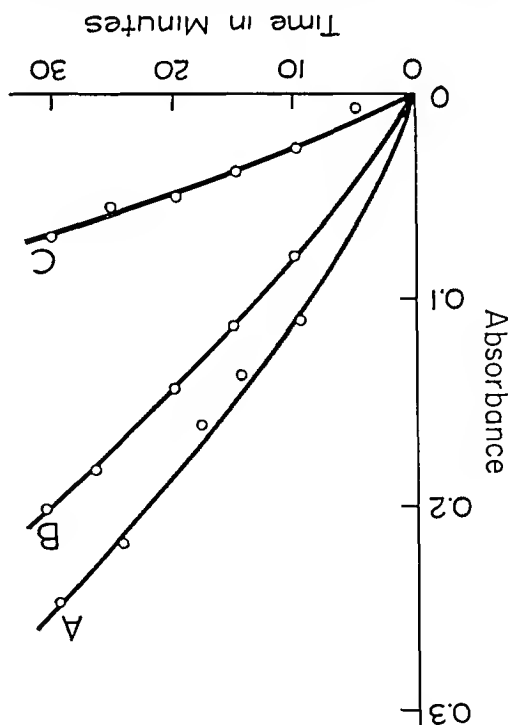
accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome. This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24). In this disease, it is possible that 8-MOP may act in several ways: (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation. All of these speculations remain to be proved.

## REFERENCES

- (1) Fitzpatrick, T. B., and Pathak, M. A., *J. Invest. Dermatol.*, 32, 229 (1959).
- (2) London, I. D., *ibid.*, 32, 315 (1959).
- (3) Jarett, A., and Szabo, C., *Brit. J. Dermatol.*, 68, 313 (1956).
- (4) Stegmayer, O. C., *J. Invest. Dermatol.*, 32, 345 (1959).
- (5) Daniels, F., Hopkins, C. E., Imbrey, J. D., Bergeron, T., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, 32, 321 (1959).
- (6) Kanof, N. B., *ibid.*, 24, 5 (1955).
- (7) Levan, H. M., *Am. J. Med. Sci.*, 190, 902 (1955).
- (8) Levan, H. M., *Quart. Bull. Northwestern Univ. Med. School*, 30, 1 (1956).
- (9) Fitzpatrick, T. B., and Szabo, C., *J. Invest. Dermatol.*, 32, 197 (1959).
- (10) Lerner, A. B., and Case, J. D., *ibid.*, 32, 211 (1959).
- (11) Lerner, A. B., Denton, C. R., and Fitzpatrick, T. B., *ibid.*, 20, 299 (1953).
- (12) Rowlands, W. L., *ibid.*, 32, 249 (1959).
- (13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330.
- (14) Hirsch, H. M., *Cancer Research*, 16, 1076 (1956).
- (15) Moller, C., Wassman, H. A., and Williams, J. N., *J. Arch. Biochem. Biophys.*, 72, 253 (1957).
- (16) Moller, C., Wassman, H. A., and Williams, J. N., *ibid.*, 72, 271 (1957).
- (17) Moller, C., Williams, H. A., and Wassman, H. A., *ibid.*, 75, 46 (1958).
- (18) Hirsch, H. M., *Radiation Research*, 5, 9 (1956).
- (19) Isaka, S., and N'Nomo, M., *Nature*, 177, 184 (1956).
- (20) Lerner, A. B., and Fitzpatrick, T. B., *Calcins*, 15, and Summerson, W. H., *J. Biol. Chem.*, 187, 793 (1950).
- (21) Daniels, F., *J. Invest. Dermatol.*, 32, 147 (1959).
- (22) Lerner, A. B., *ibid.*, 32, 285 (1959).
- (23) Lerner, A. B., *ibid.*, 32, 285 (1959).

Fig. 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light." A, pH 8.05; B, pH 6.6; C, pH 5.8. Absorbance was measured at 480 mμ.



groups. Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation. Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation. While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals. It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation. A couple of compounds believed to be radioprotective by a chelating mechanism were also included. Table I gives the results obtained.

It is well known that in the normal sustaining process, melanin formation is stimulated (8, 9, 22). Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known. Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8). The mechanism by which 8-MOP may stimulate the sustaining process could possibly be due to its

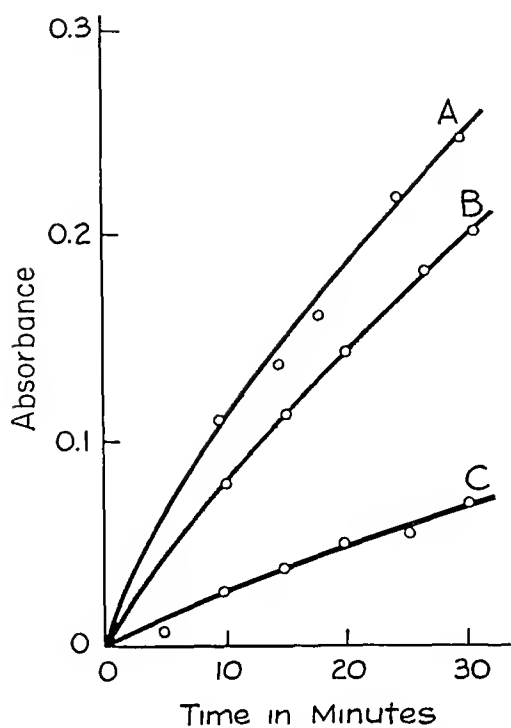


Fig 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light" A, pH 8.05, B, pH 6.6, C, pH 5.3 Absorbance was measured at 480 mμ.

groups Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation A couple of compounds believed to be radioprotective by a chelating mechanism were also included Table I gives the results obtained

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22) Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8) The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit <sup>a</sup>
KCN	0.03 M	—
Disodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>b</sup>	0.01 M	+
Cysteamine HCl	0.01 M	+
AET	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg of 8 MOP, 1 mg of DOPA, 1 ml of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 M phosphate buffer, and enough phosphate buffer to make 6.0 ml

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome This is presumably due to the photo-oxidation of sodium thioglycollate

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24) In this disease, it is possible that 8-MOP may act in several ways (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation All of these speculations remain to be proved

REFERENCES

(1) Fitzpatrick, T. B., and Pathak, M. A., *J. Invest. Dermatol.*, **32**, 229 (1959)  
(2) London, I. D., *ibid.*, **32**, 315 (1959)  
(3) Jarrett, A., and Szabo, G., *Brit. J. Dermatol.*, **68**, 313 (1956)  
(4) Stegmaier, O. C., *J. Invest. Dermatol.*, **32**, 345 (1959)  
(5) Daniels, F., Jr., Hopkins, C. E., Imbrie, J. D., Bergeron, L., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, **32**, 321 (1959)  
(6) Kanof, N. B., *ibid.*, **24**, 5 (1955)  
(7) Lerner, A. B., *Am. J. Med.*, **19**, 902 (1955)  
(8) Levin, H. M., *Quart. Bull. Northwestern Univ. Med. School*, **30**, 1 (1956)  
(9) Fitzpatrick, T. B., and Szabo, G., *J. Invest. Dermatol.*, **32**, 197 (1959)  
(10) Lerner, A. B., and Case, J. D., *ibid.*, **32**, 211 (1959)  
(11) Lerner, A. B., Denton, C. R., and Fitzpatrick, T. B., *ibid.*, **20**, 299 (1953)  
(12) Fowles, W. L., *ibid.*, **32**, 249 (1959)  
(13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330  
(14) Hirsch, H. M., *Cancer Research*, **16**, 1076 (1956)  
(15) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *Arch. Biochem. Biophys.*, **72**, 255 (1957)  
(16) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *ibid.*, **72**, 271 (1957)  
(17) Monder, C., Williams, J. N., Jr., and Waisman, H. A., *ibid.*, **75**, 46 (1958)  
(18) Hirsch, H. M., *Radiation Research*, **5**, 9 (1956)  
(19) Isaka, S., and Akino, M., *Nature*, **177**, 184 (1956)  
(20) Isaka, S., *ibid.*, **179**, 578 (1957)  
(21) Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H., *J. Biol. Chem.*, **187**, 793 (1950)  
(22) Daniels, F., Jr., *J. Invest. Dermatol.*, **32**, 147 (1959)  
(23) Pinkus, H., *ibid.*, **32**, 281 (1959)  
(24) Lerner, A. B., *ibid.*, **32**, 285 (1959)

# Effects of Fatty Acids on Vitamin A Esters in Isopropanol Solutions\*

By ALBERT J. FORLANO† and LOYD E. HARRIS

Vitamin A ester degradation was studied in isopropyl alcohol, isopropyl alcohol and water, cyclohexane, and in these solvents containing members of the acetic acid series of fatty acids. The results indicate that (a) the principal methods of degradation are oxidation and elimination in hydroxylated solvents, (b) water increases the rates of elimination, (c) fatty acids decrease the rates of elimination, and (d) oxidation is the main route of decomposition in hydrocarbon solvents. The stabilizing mechanism of the fatty acids was studied by examining the effects of fatty acids, water, and isopropyl alcohol on anhydrovitamin A.

VITAMIN A esters were unstable in vanishing creams containing stearic acid. The nature of this instability has not been established. The study reported herein was undertaken to determine whether fatty acids caused this instability and the nature of the decomposition. Since vitamin A is known to be sensitive to mineral acids (1, 2), it was assumed that stearic acid was the causative agent. That the rate was slower in the presence of fatty acids appeared to be related to the smaller ( $H^+$ ) liberated by the fatty acids.

Vitamin A palmitate degradation was initially studied in *n*-hexane and isopropanol, both containing 5 per cent stearic acid. In *n*-hexane vitamin A was lost mainly through oxidation; however in isopropanol, both elimination and oxidation occurred. A plot of log concentration versus time was a straight line indicative of a first-order dependence on the vitamin A concentration. Some samples were chromatographed on Woelm alumina, deactivated by the addition of 6 per cent water.

## EXPERIMENTAL

**Materials Used in This Study.**—Isopropanol (81–83°) Sohio; Fisher reagent grade stearic acid (68–69.5°); Fisher reagent grade palmitic acid (61–62°); Fisher laboratory chemicals oleic and caproic acids (purified); Eastman Kodak decanoic acid; Dupont reagent glacial acetic acid (99.7%); Mallinckrodt anhydrous ethyl ether; heavy liquid petrolatum U. S. P.; *n*-hexane (technical) OSU label (redistilled); Woelm neutral activated alumina for chromatography (M. Woelm, Eschwage, Germany); vitamin A acetate and palmitate, Hoffmann-La Roche, Inc.; and butylated hydroxy toluene (BHT), food grade, Koppers Co., Inc.

**Stability of Vitamin A in the Presence of a Homologous Series of Fatty Acids in Isopropanol.**—The

purpose of this study was to determine (a) if they would accelerate destruction of vitamin A esters, (b) if there was any difference in the rates of destruction among the members of a homologous series of fatty acids, (c) the nature of decomposition.

Isopropanol was chosen as the solvent because it is water miscible, good solvent for vitamin A esters and the fatty acids, and had only a slight destructive action on vitamin A (3). In order to determine the rates of decomposition and order of dependence on vitamin A, the other ingredients were in a large excess of the vitamin A concentration. Fatty acid concentrations of 0.033 *N* were used because mineral acids in the same concentrations were effective in causing elimination of vitamin A esters and alcohol (2).

**Preparation of Solutions.**—The distilled isopropanol was flushed with dry nitrogen for one hour. The water content of this solvent was calculated at 0.056% by a Karl Fischer titration (4). Calculated quantities of caproic, decanoic, palmitic, stearic, and oleic acids were added to make 0.033 *N* solutions and enough vitamin A palmitate was added to make an approximate concentration of 10,000 u./ml. A control, not containing fatty acid, was also prepared. The solutions were placed into 120-ml. glass-stoppered amber bottles and their vitamin A potency was determined by the British Pharmacopoeia method (5). The air in these containers was displaced by nitrogen. These containers were subsequently stored at 45, 37, and 25°. When these solutions were titrated with base, it was found that none of the fatty acids were lost through esterification with the isopropanol. The U. V. absorption of the fatty acids did not interfere with the spectrum of the vitamin A. The rates of decomposition are given in Table I. Figure 1 represents the plot of log concentration of vitamin A versus time. The control graph is submitted as representative of the entire group.

This graph indicates a pseudo first-order reaction depending only on the concentration of vitamin A ester. The rate constants, except for a few cases ( $k_1$  for stearic, palmitic, and oleic acids at 45° only), indicated that the rate of destruction was slower in the presence of fatty acids and also that the amount of anhydrovitamin A was smaller in the presence of fatty acids. The unexpected drop in the 45° curve (Fig. 1) was due to oxidation which was seen in the entire series. The first three hundred hours could well be considered an induction period before oxidation began. The U. V. extinction at

\* Received August 21, 1959, from the College of Pharmacy, Ohio State University, Columbus 10.

Based, in part, upon a Ph.D. dissertation submitted to the Graduate School, Ohio State University.

† Present address: Chas. Pfizer & Co., Inc., Brooklyn, N.Y.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.



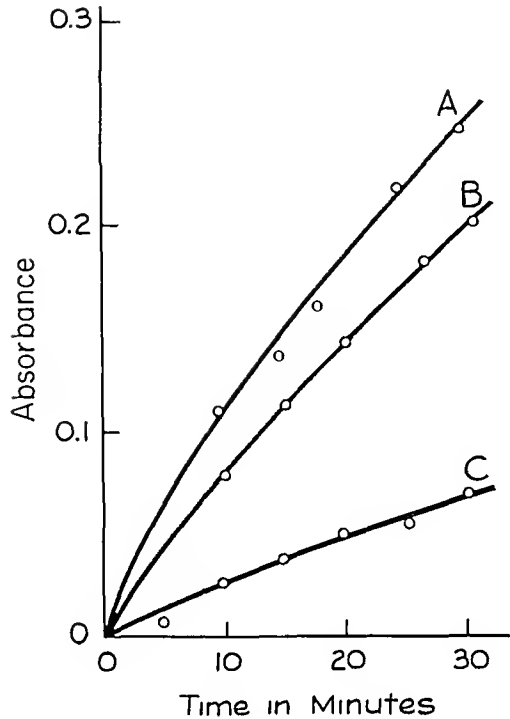


Fig 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light" A, pH 8.05, B, pH 6.6, C, pH 5.3 Absorbance was measured at 480 mμ.

groups Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation A couple of compounds believed to be radioprotective by a chelating mechanism were also included Table I gives the results obtained

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22) Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8) The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit <sup>a</sup>
KCN	0.03 M	—
Disodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>b</sup>	0.01 M	+
Cysteamine HCl	0.01 M	+
AET	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol of an appropriate amount of the substance dissolved in pH 6.3, 0.067 M phosphate buffer and enough phosphate buffer to make 6.0 ml.

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome.

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation the inhibition was almost completely overcome This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24) In this disease, it is possible that 8-MOP may act in several ways (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation All of these speculations remain to be proved

REFERENCES

(1) Fitzpatrick, T. B., and Pathak, M. A., *J. Invest. Dermatol.*, **32**, 229 (1959).  
(2) London, I. D., *ibid.*, **32**, 315 (1959).  
(3) Jarrett, A., and Szabo, G., *Brit. J. Dermatol.*, **68**, 315 (1956).  
(4) Stegmaier, O. C., *J. Invest. Dermatol.*, **32**, 345 (1959).  
(5) Daniels, F., Jr., Hopkins, C. E., Imbrie, J. D., Bergeron, L., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, **32**, 321 (1959).  
(6) Kanof, N. B., *ibid.*, **24**, 5 (1955).  
(7) Lerner, A. B., *Am. J. Med.*, **19**, 902 (1955).  
(8) Levin, H. M., *Quart. Bull. Northwestern Univ. Med. School*, **30**, 1 (1956).  
(9) Fitzpatrick, T. B., and Szabo, G., *J. Invest. Dermatol.*, **32**, 197 (1959).  
(10) Lerner, A. B., and Case, J. D., *ibid.*, **32**, 211 (1959).  
(11) Lerner, A. B., Denton, C. R., and Fitzpatrick, T. B., *ibid.*, **20**, 299 (1953).  
(12) Fowles, W. L., *ibid.*, **32**, 249 (1959).  
(13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330.  
(14) Hirsch, H. M., *Cancer Research*, **16**, 1076 (1956).  
(15) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *Arch. Biochem. Biophys.*, **72**, 255 (1957).  
(16) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *ibid.*, **72**, 271 (1957).  
(17) Monder, C., Williams, J. N., Jr., and Waisman, H. A., *ibid.*, **75**, 46 (1958).  
(18) Hirsch, H. M., *Radiation Research*, **5**, 9 (1956).  
(19) Isaka, S., and Akino, M., *Nature*, **177**, 184 (1956).  
(20) Isaka, S., *ibid.*, **179**, 578 (1957).  
(21) Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson, W. H., *J. Biol. Chem.*, **187**, 793 (1950).  
(22) Daniels, F., Jr., *J. Invest. Dermatol.*, **32**, 147 (1959).  
(23) Pinkus, H., *ibid.*, **32**, 281 (1959).  
(24) Lerner, A. B., *ibid.*, **32**, 285 (1959).

# Effects of Fatty Acids on Vitamin A Esters in Isopropanol Solutions\*

By ALBERT J. FORLANO† and LOYD E. HARRIS

Vitamin A ester degradation was studied in isopropyl alcohol, isopropyl alcohol and water, cyclohexane, and in these solvents containing members of the acetic acid series of fatty acids. The results indicate that (a) the principal methods of degradation are oxidation and elimination in hydroxylated solvents, (b) water increases the rates of elimination, (c) fatty acids decrease the rates of elimination, and (d) oxidation is the main route of decomposition in hydrocarbon solvents. The stabilizing mechanism of the fatty acids was studied by examining the effects of fatty acids, water, and isopropyl alcohol on anhydrovitamin A.

VITAMIN A esters were unstable in vanishing creams containing stearic acid. The nature of this instability has not been established. The study reported herein was undertaken to determine whether fatty acids caused this instability and the nature of the decomposition. Since vitamin A is known to be sensitive to mineral acids (1, 2), it was assumed that stearic acid was the causative agent. That the rate was slower in the presence of fatty acids appeared to be related to the smaller ( $H^+$ ) liberated by the fatty acids.

Vitamin A palmitate degradation was initially studied in *n*-hexane and isopropanol, both containing 5 per cent stearic acid. In *n*-hexane vitamin A was lost mainly through oxidation; however in isopropanol, both elimination and oxidation occurred. A plot of log concentration versus time was a straight line indicative of a first-order dependence on the vitamin A concentration. Some samples were chromatographed on Woelm alumina, deactivated by the addition of 6 per cent water.

## EXPERIMENTAL

**Materials Used in This Study.**—Isopropanol (81–83°) Sohio; Fisher reagent grade stearic acid (68–69.5°); Fisher reagent grade palmitic acid (61–62°); Fisher laboratory chemicals oleic and caproic acids (purified); Eastman Kodak decanoic acid; Dupont reagent glacial acetic acid (99.7%); Mallinckrodt anhydrous ethyl ether; heavy liquid petrolatum U. S. P.; *n*-hexane (technical) OSU label (redistilled); Woelm neutral activated alumina for chromatography (M. Woelm, Eschwage, Germany); vitamin A acetate and palmitate, Hoffmann-La Roche, Inc.; and butylated hydroxy toluene (BHT), food grade, Koppers Co., Inc.

**Stability of Vitamin A in the Presence of a Homologous Series of Fatty Acids in Isopropanol.**—The

purpose of this study was to determine (a) if they would accelerate destruction of vitamin A esters, (b) if there was any difference in the rates of destruction among the members of a homologous series of fatty acids, (c) the nature of decomposition.

Isopropanol was chosen as the solvent because it is water miscible, good solvent for vitamin A esters and the fatty acids, and had only a slight destructive action on vitamin A (3). In order to determine the rates of decomposition and order of dependence on vitamin A, the other ingredients were in a large excess of the vitamin A concentration. Fatty acid concentrations of 0.033 *N* were used because mineral acids in the same concentrations were effective in causing elimination of vitamin A esters and alcohol (2).

**Preparation of Solutions.**—The distilled isopropanol was flushed with dry nitrogen for one hour. The water content of this solvent was calculated at 0.056% by a Karl Fischer titration (4). Calculated quantities of caproic, decanoic, palmitic, stearic, and oleic acids were added to make 0.033 *N* solutions and enough vitamin A palmitate was added to make an approximate concentration of 10,000 u./ml. A control, not containing fatty acid, was also prepared. The solutions were placed into 120-ml. glass-stoppered amber bottles and their vitamin A potency was determined by the British Pharmacopoeia method (5). The air in these containers was displaced by nitrogen. These containers were subsequently stored at 45, 37, and 25°. When these solutions were titrated with base, it was found that none of the fatty acids were lost through esterification with the isopropanol. The U. V. absorption of the fatty acids did not interfere with the spectrum of the vitamin A. The rates of decomposition are given in Table I. Figure 1 represents the plot of log concentration of vitamin A versus time. The control graph is submitted as representative of the entire group.

This graph indicates a pseudo first-order reaction depending only on the concentration of vitamin A ester. The rate constants, except for a few cases ( $k_1$  for stearic, palmitic, and oleic acids at 45° only), indicated that the rate of destruction was slower in the presence of fatty acids and also that the amount of anhydrovitamin A was smaller in the presence of fatty acids. The unexpected drop in the 45° curve (Fig. 1) was due to oxidation which was seen in the entire series. The first three hundred hours could well be considered an induction period before oxidation began. The U. V. extinction at

\* Received August 21, 1959, from the College of Pharmacy, Ohio State University, Columbus 10.

Based, in part, upon a Ph.D. dissertation submitted to the Graduate School, Ohio State University.

† Present address: Chas. Pfizer & Co., Inc., Brooklyn, N.Y.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

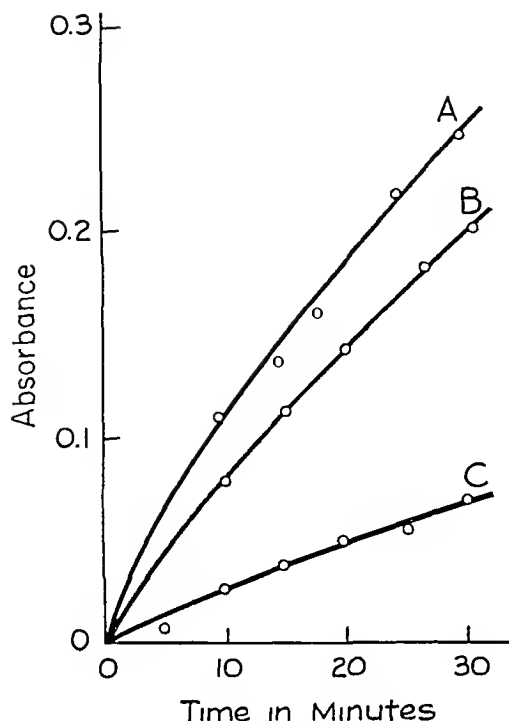


Fig 5—The effect of pH on photo-oxidation of DOPA by 8-MOP in "black light" A, pH 8.05, B, pH 6.6, C, pH 5.3 Absorbance was measured at 480  $\mu$ .

groups. Substances or conditions preserving the latter inhibit oxidation and the reverse stimulate oxidation. Hirsch (13, 18) in addition, has pointed out that agents inhibiting radiation induced DOPA oxidation may operate by one or both of two mechanisms: (a) by complexing copper which is an integral part of tyrosinase and which also stimulates DOPA oxidation *in vitro* (19, 20, 21) and (b) by destroying oxidizing radicals such as the hydroxyl and perhydroxyl radicals that would arise from the action of ionizing radiations in aqueous media in radiation-induced DOPA oxidation. While the precise mechanism of the photochemical or photodynamic effect is not known, it is possible that the actual oxidation of the substrate may be due to the oxidizing radicals. It therefore seemed worth while to determine whether radioprotective compounds which are believed to reduce these oxidizing radicals would also protect DOPA from photo-oxidation. A couple of compounds believed to be radioprotective by a chelating mechanism were also included. Table I gives the results obtained.

It is well known that in the normal suntanning process, melanin formation is stimulated (8, 9, 22). Whether new melanin is produced or previously-formed but reduced melanin is oxidized is not known. Nevertheless, it has been shown that sulfhydryl groups inhibit melanin formation (7, 8). The mechanism by which 8-MOP may stimulate the suntanning process could possibly be due to its

TABLE I.—EFFECT OF VARIOUS SUBSTANCES ON THE PHOTO-OXIDATION OF DOPA BY 8-MOP IN "BLACK LIGHT"

Substance	Final Concentration	Ability to Inhibit <sup>a</sup>
KCN	0.03 M	—
Disodium versenate	0.02 M	—
Ascorbic acid	0.002 M	+
Cysteine HCl	0.005 M	+
Sodium thioglycollate <sup>b</sup>	0.01 M	+
Cysteamine HCl	0.01 M	+
AET	0.01 M	+

<sup>a</sup> The reaction mixtures contained 1 mg. of 8-MOP, 1 mg. of DOPA, 1 ml. of ethanol, an appropriate amount of the test substance dissolved in pH 6.3, 0.067 M phosphate buffer, and enough phosphate buffer to make 6.0 ml.

<sup>b</sup> Sodium thioglycollate caused complete inhibition for thirty minutes, but by fifty minutes of irradiation, the inhibition was completely overcome.

accumulation in the melanocytes where it would photochemically oxidize sulfhydryl groups, thus allowing melanin formation to proceed. In the experiment involving inhibitors of the photo-oxidation of DOPA, sodium thioglycollate inhibited for thirty minutes but by fifty minutes of irradiation, the inhibition was almost completely overcome. This is presumably due to the photo-oxidation of sodium thioglycollate.

In vitiligo, the abnormality seems to be strictly a functional one of the melanocyte (23, 24). In this disease, it is possible that 8-MOP may act in several ways: (a) to photo-oxidize some inhibitor of melanin formation which is present in abnormal amounts in the vitiliginous area, (b) to photo-oxidize any quantities of DOPA present to melanin, (c) to raise the oxidation-reduction potential making the conditions more favorable for normal melanin formation. All of these speculations remain to be proved.

## REFERENCES

- (1) Fitzpatrick, T. B., and Pathak, M. A., *J. Invest. Dermatol.*, 32, 229 (1959).
- (2) London, I. D., *ibid.*, 32, 315 (1959).
- (3) Jarrett, A., and Szabo, G., *Brit. J. Dermatol.*, 68, 313 (1956).
- (4) Stegmayer, O. C., *J. Invest. Dermatol.*, 32, 345 (1959).
- (5) Daniels, F., Jr., Hopkins, C. E., Imbrie, J. D., Bergeron, L., Miller, O., Crowe, F., and Fitzpatrick, T. B., *ibid.*, 32, 321 (1959).
- (6) Kanof, N. B., *ibid.*, 24, 5 (1955).
- (7) Lerner, A. B., *Am. J. Med.*, 19, 902 (1955).
- (8) Levin, H. M., *Quart. Bull. Northwestern Univ. Med. School*, 30, 1 (1956).
- (9) Fitzpatrick, T. B., and Szabo, G., *J. Invest. Dermatol.*, 32, 197 (1959).
- (10) Lerner, A. B., and Case, J. D., *ibid.*, 32, 211 (1959).
- (11) Lerner, A. B., Denton, C. R., and Fitzpatrick, T. B., *ibid.*, 20, 299 (1953).
- (12) Daniels, F., Jr., *ibid.*, 32, 249 (1959).
- (13) Hirsch, H. M., "Pigment Cell Biology," Academic Press Inc., New York, N. Y., 1959, p. 330.
- (14) Hirsch, H. M., *Cancer Research*, 16, 1076 (1956).
- (15) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *Arch. Biochem. Biophys.*, 72, 255 (1957).
- (16) Monder, C., Waisman, H. A., and Williams, J. N., Jr., *ibid.*, 72, 271 (1957).
- (17) Monder, C., Williams, J. N., Jr., and Waisman, H. A., *ibid.*, 75, 46 (1958).
- (18) Hirsch, H. M., *Radiation Research*, 5, 9 (1956).
- (19) Isaka, S., and Akino, M., *Nature*, 177, 181 (1956).
- (20) Isaka, S., *ibid.*, 179, 578 (1957).
- (21) Lerner, A. B., Fitzpatrick, T. B., Calkins, E., and Summerson W. H., *J. Biol. Chem.*, 187, 793 (1950).
- (22) Daniels, F., Jr., *J. Invest. Dermatol.*, 32, 147 (1959).
- (23) Pinkus, H., *ibid.*, 32, 281 (1959).
- (24) Lerner, A. B., *ibid.*, 32, 285 (1959).

# Effects of Fatty Acids on Vitamin A Esters in Isopropanol Solutions\*

By ALBERT J. FORLANO† and LOYD E. HARRIS

Vitamin A ester degradation was studied in isopropyl alcohol, isopropyl alcohol and water, cyclohexane, and in these solvents containing members of the acetic acid series of fatty acids. The results indicate that (a) the principal methods of degradation are oxidation and elimination in hydroxylated solvents, (b) water increases the rates of elimination, (c) fatty acids decrease the rates of elimination, and (d) oxidation is the main route of decomposition in hydrocarbon solvents. The stabilizing mechanism of the fatty acids was studied by examining the effects of fatty acids, water, and isopropyl alcohol on anhydrovitamin A.

VITAMIN A esters were unstable in vanishing creams containing stearic acid. The nature of this instability has not been established. The study reported herein was undertaken to determine whether fatty acids caused this instability and the nature of the decomposition. Since vitamin A is known to be sensitive to mineral acids (1, 2), it was assumed that stearic acid was the causative agent. That the rate was slower in the presence of fatty acids appeared to be related to the smaller ( $H^+$ ) liberated by the fatty acids.

Vitamin A palmitate degradation was initially studied in *n*-hexane and isopropanol, both containing 5 per cent stearic acid. In *n*-hexane vitamin A was lost mainly through oxidation; however in isopropanol, both elimination and oxidation occurred. A plot of log concentration versus time was a straight line indicative of a first-order dependence on the vitamin A concentration. Some samples were chromatographed on Woelm alumina, deactivated by the addition of 6 per cent water.

## EXPERIMENTAL

**Materials Used in This Study.**—Isopropanol (81–83°) Sohio; Fisher reagent grade stearic acid (68–69.5°); Fisher reagent grade palmitic acid (61–62°); Fisher laboratory chemicals oleic and caproic acids (purified); Eastman Kodak decanoic acid; Dupont reagent glacial acetic acid (99.7%); Mallinckrodt anhydrous ethyl ether; heavy liquid petrolatum U. S. P.; *n*-hexane (technical) OSU label (redistilled); Woelm neutral activated alumina for chromatography (M. Woelm, Eschwage, Germany); vitamin A acetate and palmitate, Hoffmann-La Roche, Inc.; and butylated hydroxy toluene (BHT), food grade, Koppers Co., Inc.

**Stability of Vitamin A in the Presence of a Homologous Series of Fatty Acids in Isopropanol.**—The

purpose of this study was to determine (a) if they would accelerate destruction of vitamin A esters, (b) if there was any difference in the rates of destruction among the members of a homologous series of fatty acids, (c) the nature of decomposition.

Isopropanol was chosen as the solvent because it is water miscible, good solvent for vitamin A esters and the fatty acids, and had only a slight destructive action on vitamin A (3). In order to determine the rates of decomposition and order of dependence on vitamin A, the other ingredients were in a large excess of the vitamin A concentration. Fatty acid concentrations of 0.033*N* were used because mineral acids in the same concentrations were effective in causing elimination of vitamin A esters and alcohol (2).

**Preparation of Solutions.**—The distilled isopropanol was flushed with dry nitrogen for one hour. The water content of this solvent was calculated at 0.056% by a Karl Fischer titration (4). Calculated quantities of caproic, decanoic, palmitic, stearic, and oleic acids were added to make 0.033 *N* solutions and enough vitamin A palmitate was added to make an approximate concentration of 10,000 u./ml. A control, not containing fatty acid, was also prepared. The solutions were placed into 120-ml. glass-stoppered amber bottles and their vitamin A potency was determined by the British Pharmacopoeia method (5). The air in these containers was displaced by nitrogen. These containers were subsequently stored at 45, 37, and 25°. When these solutions were titrated with base, it was found that none of the fatty acids were lost through esterification with the isopropanol. The U. V. absorption of the fatty acids did not interfere with the spectrum of the vitamin A. The rates of decomposition are given in Table I. Figure 1 represents the plot of log concentration of vitamin A versus time. The control graph is submitted as representative of the entire group.

This graph indicates a pseudo first-order reaction depending only on the concentration of vitamin A ester. The rate constants, except for a few cases ( $k_1$  for stearic, palmitic, and oleic acids at 45° only), indicated that the rate of destruction was slower in the presence of fatty acids and also that the amount of anhydrovitamin A was smaller in the presence of fatty acids. The unexpected drop in the 45° curve (Fig. 1) was due to oxidation which was seen in the entire series. The first three hundred hours could well be considered an induction period before oxidation began. The U. V. extinction at

\* Received August 21, 1959, from the College of Pharmacy, Ohio State University, Columbus 10.

Based, in part, upon a Ph.D. dissertation submitted to the Graduate School, Ohio State University.

† Present address: Chas. Pfizer & Co., Inc., Brooklyn, N.Y.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

TABLE I.—RATE CONSTANTS FOR DEGRADATION OF VITAMIN A IN ISOPROPANOL SOLUTION  
( $k/2.303 \text{ hr}^{-1} \times 10^4$ )

	Isopropanol Control	0.033 N Caproic Acid	0.033 N Decanoic Acid	0.033 N Palmitic Acid	0.033 N Oleic Acid	0.033 N Stearic Acid
45°						
$k_1$	13.7	12.0	12.5	14.9	15.0	15.0
$k_2$	1.50	1.00	1.13	1.05	1.33	1.25
37°						
$k_1$	12.0	7.70	5.30	5.90	7.01	8.00
$k_2$	1.25	0.980	0.938	0.833	0.910	0.834

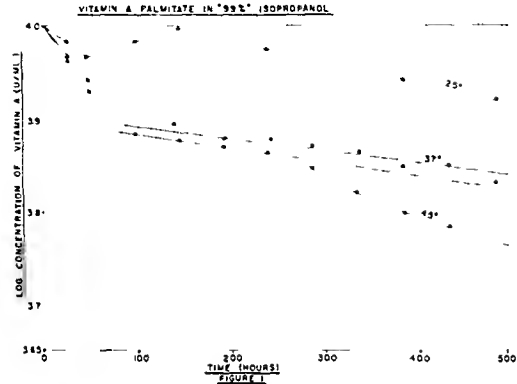


Fig. 1.—Vitamin A palmitate in "99%" isopropanol.

280  $\mu$  rose sharply at the same time the unexpected drop in potency was observed. The lump in the 25° curve is unexplainable even after careful consideration of the data collected.

**Chromatography.**—The reaction mixtures were chromatographed on Woelm alumina deactivated by the addition of 6% water. Ninety-four grams of activated alumina was placed in a glass-stoppered flask containing 6 Gm. of water and the mixture was allowed to hydrate for two hours, enough purified *n*-hexane was added to the alumina to make a slurry. This slurry was poured into a glass column to a height of 10 inches of alumina.

The isopropanol reaction mixtures were dried *in vacuo* and the residue was dissolved in 5 ml. of purified *n*-hexane. The solution was poured into the column and was immediately followed by *n*-hexane so that the column would not dry out. The weakest eluant, *n*-hexane, was added first and 25-ml. fractions were collected. Anhydrovitamin A, the first substance eluted, was followed by vitamin A esters, both being eluted by *n*-hexane. The following eluate fractions contained very little material as determined by the U. V. absorption; consequently it was necessary to use stronger eluants (2, 4, 8, 16, 25, 40, 50, and 75% ethyl ether in *n*-hexane) to remove the substances remaining on the column.

The U. V. spectra of each of these fractions were determined on a Beckman DK spectrophotometer. Each fraction was subsequently dried *in vacuo* and their I. R. spectra were determined using a Baird infrared spectrophotometer between sodium chloride plates.

The following substances were identified and were eluted in this order: anhydrovitamin A (max. 351  $\mu$ , 371  $\mu$ , and 391  $\mu$ ); vitamin A

esters (max. 326  $\mu$ ); esterified oxidation products (max. 285  $\mu$ ); vitamin A alcohol (max. 325  $\mu$ ); rehydro substance (max. 333  $\mu$ , 348  $\mu$ , and 369  $\mu$ ); oxidation product (max. 280  $\mu$ ); oxidation product (max. 280  $\mu$  and below 235  $\mu$ ).

**Effects of Addition of Water to the Isopropanol Samples.**—Since the fatty acids had a stabilizing action on vitamin A palmitate in isopropanol, 5% of distilled water was added to the isopropanol to observe the effects of increased ionization of the fatty acids. The exact water content was 4.66% by Karl Fischer titration (4). This solvent will be referred to as "95%" isopropanol. The solutions of vitamin A esters were prepared and treated in the same fashion as the previous series except that vitamin A acetate was substituted for vitamin A palmitate because the latter was not sufficiently soluble in "95%" isopropanol.

A new assay method (6) was adopted because the "95%" isopropanol was not miscible with the cyclohexane of the B. P. method which is used as the diluting solvent.

$$E_1 = 314 \mu, \quad E_2 = 326 \mu, \quad E_3 = 338 \mu$$
$$E(1\%, 1 \text{ cm.}) (\text{corr.}) = \frac{3.61 (2E_2 - E_1 - E_3)}{\% \text{ dilution} \times \text{length of cell (cm.)}}$$

$$\text{Potency u./ml.} = E(1\%, 1 \text{ cm.}) \times 1900$$

The rates were measured over a period of one hundred and twenty hours at which time the 45° samples showed 60% loss. The rates of degradation are given in Table II.

TABLE II.—RATE CONSTANTS FOR THE DEGRADATION OF VITAMIN A IN "95%" ISOPROPANOL SOLUTION  
( $k/2.303 \times \text{hr.}^{-1} \times 10^4$ )

	45°	37°	25°
Control (no fatty acid)	41.7	14.3	3.9
0.033 N Caproic acid	23.5	7.7	2.4
0.033 N Decanoic acid	24.5	10.1	4.1
0.033 N Palmitic acid	24.0	8.3	2.6
0.033 N Oleic acid	25.0	7.2	2.7
0.033 N Stearic acid	27.9	10.0	5.6

It appeared that in this case the fatty acids preserved the vitamin A potency and maintained a lower concentration of anhydrovitamin A, whereas water had a catalytic effect on vitamin A destruction.

A plot of log concentration versus time of the control series is submitted in Fig. 2.

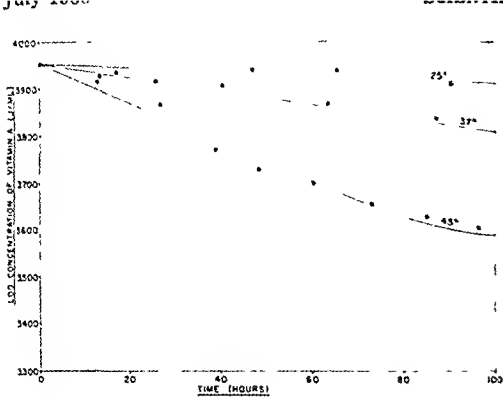


Fig. 2.—Vitamin A acetate in "95%" isopropanol.

Since it was shown that the extinction at 391  $\mu$  could be used to determine the amount of anhydrovitamin A present (1) the following formula was derived to convert  $E_{391} \mu$  into the original units of vitamin A from which it was derived.

$$E_{391} \mu \times 1115 = \text{anhydrovitamin A}$$

Calculations based on this formula showed that approximately 50% of the decomposition was of an eliminative nature.

**Alumina Chromatography of the Samples.**—The control, 0.033 *N* palmitic and caproic acid samples at 45° were chromatographed on alumina according to the procedure described under the alumina chromatography of "99%" isopropanol series.

Since many of the fractions contained identical substances it was not necessary to subject each fraction of U. V. and I. R. spectroscopy. A plot of U. V. absorbance versus fraction number gave a good indication as to when a new substance was being eluted. The center fraction of each group on the graph was chosen as the representative of the particular group as shown in Fig. 3. This fraction was subjected to U. V. and I. R. spectroscopy.

The following substances were eluted in the order mentioned: anhydrovitamin A (max. 351  $\mu$ , 371  $\mu$ , and 391  $\mu$ ); vitamin A ester (max. 326  $\mu$ ); esterified oxidation products (max. 290  $\mu$ ); rehydro structure (max. 328  $\mu$ , 348  $\mu$ , and 368  $\mu$ ); vitamin A alcohol (max. 325  $\mu$ ); oxidation products (max. 288–290  $\mu$ ).

**Paper Chromatography.**—The representative fractions were chromatographed on paper to determine if they were single substances or mixtures. Whatman filter paper No. 1 (1½-in. strips) were impregnated with 6% liquid petrolatum in petroleum ether (30–60°) and the solvent was allowed to evaporate. A drop of the sample was placed on the starting line and the strips were placed in a tank saturated with ethanol and nitrogen vapors. The strips were allowed to develop for seven to eight hours with ethanol U. S. P. as the eluant. The zones were identified by their fluorescence in U. V. light and by eluting the material from the paper with ethanol U. S. P. and determining their spectra. The controls had the following  $R_f$  values: vitamin A palmitate 0.090; acetate 0.514; and alcohol 0.895. Based on these  $R_f$  values all the samples contained vitamin A alcohol, ester (originally introduced),

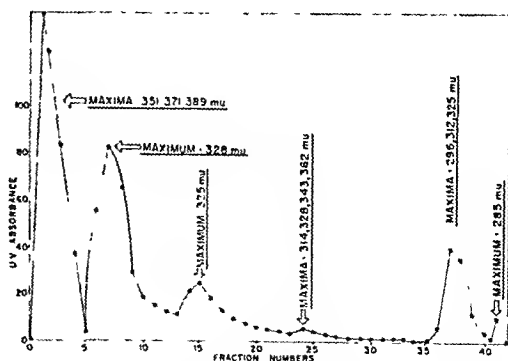


Fig. 3.—Chromatography of "95%" isopropanol control sample (45°).

and oxidation products. The samples containing fatty acids also contained a small quantity of ester formed from the fatty acid of the media. The oxidation products had a higher  $R_f$  value than vitamin A alcohol ( $R_f$  0.952); fixed oils, as olive oils, may also be used as an impregnating agent dissolved in petroleum ether 30–60°. Some of the alumina chromatographed fractions of oxidation products divided into two fractions. This indicated a degree of uncertainty of alumina separation of oxidation products. The vitamin A component of these solutions consisted of vitamin A alcohol, acetate, and an ester derived from the fatty acid of the media. This was significant because only vitamin A acetate was originally introduced and there was interconversion of vitamin A forms.

**Action of Isopropanol, Water, Cyclohexane, and Fatty Acids on Anhydrovitamin A.**—Embree and Shantz (7) and Orshnick (8) prepared vitamin A active substances from the reaction of acetic, propionic, oleic, benzoic acids on anhydrovitamin A in ethanol or methanol. The products were the ethyl and methyl ethers of vitamin A. In nonalcoholic solvents the same reactants produced vitamin A esters (7).

In this work it was found that fatty acids decreased the loss of ester and also decreased anhydrovitamin A formation. It is possible that the stabilization was connected with a regeneration of a vitamin A substance by the fatty acids from anhydrovitamin A.

The solvents used in this study were cyclohexane, isopropanol (0.056% water), "95%" isopropanol (4.66% water), and "85%" isopropanol (14.36% water), as determined by the Karl Fischer titrations (4). BHT, 0.1%, was added to prevent oxidation of the vitamin A substance formed and it did not interfere with the U. V. assay of vitamin A. Cyclohexane was purified by distillation followed by chromatographing on completely activated alumina. It was transparent above 240  $\mu$ . The 81–83° fraction from isopropanol distillation was used for this work. The anhydrovitamin A was prepared according to Shantz (2) by the action of HCl on vitamin A alcohol followed by purification on alumina deactivated by the addition of 3% water.

**Calculation of Quantities of Vitamin A and Anhydrovitamin A.**—A system was devised to determine the amount of vitamin A and anhydrovitamin

A present at any time 't' Since their U V spectra overlap, direct extinctions could not be used. The exact nature of vitamin A substance being formed in solution could not be determined, consequently an assumption was made that vitamin A alcohol was being formed. This was reasonable since the molar extinctions of vitamin A alcohol, ester, ether, and amine are practically equivalent. On the basis of this information, the following simultaneous equations were derived:

Anhydrovitamin A

$$\begin{aligned} E(1\% \text{ 1cm})_{351 \text{ m}\mu} &= 2500 \\ E(1\% \text{ 1cm})_{326 \text{ m}\mu} &= 700 \end{aligned} \quad \frac{1_{326}}{1_{351}} = 0.290$$

Vitamin A Alcohol

$$\begin{aligned} E(1\% \text{ 1cm})_{351 \text{ m}\mu} &= 903, \\ E(1\% \text{ 1cm})_{326 \text{ m}\mu} &= 1835 \end{aligned} \quad \frac{1_{326}}{1_{351}} = 2.032$$

Let  $x$  = extinction due to anhydrovitamin A at 351 m $\mu$ , Let  $y$  = extinction due to vitamin A at 351 m $\mu$

$$x + y = E_{351 \text{ m}\mu} \quad (\text{Eq 1})$$

$$0.290x + 2.032y = E_{326 \text{ m}\mu} \quad (\text{Eq 2})$$

solving

$$x = 1.166E_{351 \text{ m}\mu} - 0.574E_{326 \text{ m}\mu} \quad (\text{Eq 3})$$

$$y = 0.574E_{326 \text{ m}\mu} - 0.166E_{351 \text{ m}\mu} \quad (\text{Eq 4})$$

**Cyclohexane Series**—In this series only caproic and acetic acids were studied and the samples were designed to contain 2, 4, and 6 M fatty acids in cyclohexane. The anhydrovitamin A was added to these solutions to make an approximate concentration of 0.1% (0.0037 M). Each sample was divided into three parts and placed in 60 ml glass stoppered bottles which were subsequently stored at 25, 37, and 50°. A control without fatty acids was also used. The bottles were flushed with dry nitrogen every time they were opened.

The samples were assayed by diluting 1 ml of the reaction mixture to 100 ml with isopropanol, then determining the extinction at 351 m $\mu$  and 326 m $\mu$ . Equations 3 and 4 above were used to calculate the extinctions at 351 m $\mu$  due to each substance.

**Plotting the Data.**—The experiments were designed so that the fatty acids would be in a large excess of the concentration of anhydrovitamin A, consequently the order of dependence on anhydrovitamin A could be determined from an appropriate plot of the data (9). Since anhydrovitamin A was converted into substances other than vitamin A, as evidenced by the precipitation of the polymerization products, a plot of log concentration anhydrovitamin A *versus*  $t$  would not give the true rate of vitamin A substance formation.

A theoretical calculation for the extinction at 351 m $\mu$  was made, based on the assumption that all the anhydrovitamin A was converted into a vitamin A substance. The value was called " $1_{\infty}$ " and was calculated as follows:

$$\begin{aligned} \frac{E(1\% \text{ 1cm})_{351 \text{ m}\mu} \times 286^1}{E(1\% \text{ 1cm})_{\text{Anhydrovitamin A } 1 \text{ m}\mu} \times 268^1} &= \\ \frac{903 \times 286}{2500 \times 268} &= 0.386 \quad (\text{Eq 5}) \end{aligned}$$

<sup>1</sup> Molecular weights of the respective substances

$$0.386 \times E_{351 \text{ m}\mu} \text{ Anhydrovitamin A} = 1_{\infty} \quad (\text{Eq 6})$$

Another value  $1_{\infty}$ , the extinction at 351 m $\mu$  due to vitamin A substance formed at any time " $t$ " was introduced. It was believed that a plot of  $\log (1_{\infty} - 1_t)$  *versus*  $t$  would give the rate of anhydrovitamin A converted into vitamin A. This procedure helps correct for side reactions, although a small error is involved, it was considered negligible since only relative rates were desired. The plots were straight lines indicating a first order dependence on the concentration of anhydrovitamin A. No vitamin A was formed in the control without fatty acids present. These rates of conversion are found in Table III. Figure 4 represents the acetic acid samples at 50°.

TABLE III—RATES OF VITAMIN A SUBSTANCE FORMATION OF ACETIC ACID AND CAPROIC ACID—ANHYDROVITAMIN A IN CYCLOHEXANE ( $k/2.303 \times \text{hr}^{-1} \times 10^4$ )

Molarity	38°	50°
2 M acetic acid	1.25	2.60
4 M acetic acid	2.08	4.50
6 M acetic acid	3.90	7.50
2 M caproic acid	1.11	3.33
4 M caproic acid	2.37	5.85
6 M caproic acid	4.00	12.5

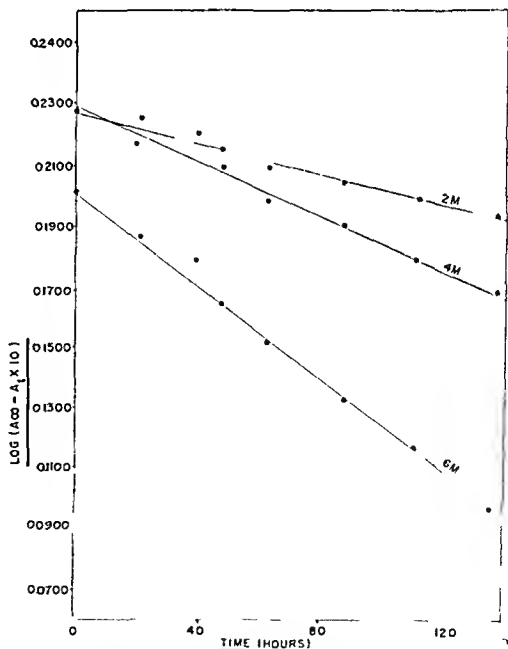


Fig 4—Rates of addition of anhydrovitamin A to acetic acid in cyclohexane at 50°

**Order of Dependence on the Fatty Acids**—A plot of  $\log k$  *versus*  $\log$  concentration of fatty acids produced lines with the following slopes: acetic acid at 50 and 37° = 1.05 and 1.09, caproic acid at 50 and 37° = 1.15 and 1.18. These data indicate a first or-

TABLE IV.—RATES FOR TRANSFORMATION OF ANHYDROVITAMIN A INTO A VITAMIN A SUBSTANCE IN "99%" ISOPROPANOL

 $(k/2.303 \times \text{hr.}^{-1} \times 10^4)$ 

	"99%" Isopropanol Control	0.033 N Acetic Acid	0.033 N Caproic Acid	0.033 N Decanoic Acid	0.033 N Palmitic Acid
50°					
$k_1$	3.00	3.75	4.50	5.25	25.0
$k_2$	1.11	1.21	1.34	1.5	2.25
37°					
$k_1$	0.81	2.21	3.12	2.5	15.0
$k_2$	0.53	0.84	0.93	1.13	2.00

der dependence on the concentration of fatty acids in this solvent system.

**Chromatography.**—The cyclohexane solutions were extracted with aqueous NaOH to remove the fatty acids, and were subsequently dried with anhydrous  $\text{Na}_2\text{SO}_4$ . The solvent was removed *in vacuo* and the residue was dissolved in 5 ml. of petroleum ether 30–60°. This solution was chromatographed on alumina using the method described under the isopropanol-vitamin A palmitate series, to isolate the vitamin A active fraction from anhydrovitamin A and oxidation products. The ester followed anhydrovitamin A from the column. The solution of the ester was evaporated *in vacuo*. The concentrate was chromatographed on paper using the method described for the vitamin A acetate in "95%" isopropanol series. The  $R_f$  values for the controls were vitamin A acetate 0.643 and anhydrovitamin A 0.320. Both samples contained anhydrovitamin A and the respective ester formed from the fatty acid of the media. The caproate ester had an  $R_f$  value of 0.506.

**Effects of Fatty Acids on Anhydrovitamin A in Isopropanol Solutions.**—The action of fatty acids on anhydrovitamin A in isopropanol, "95%" and "85%" isopropanol was studied. The preparation of the samples was similar to the cyclohexane series except that isopropanol solvents were used and the following fatty acids: acetic, caproic, decanoic, and palmitic acids, were used, only in a 0.033 N concentration. Controls without fatty acids were also used. Table IV gives the rates of conversion using isopropanol as the solvent.

It appeared that the rate of conversion varied directly with the chain length of the fatty acid and that fatty acids catalyzed the conversion. The rates of conversion for the "95%" isopropanol systems are given in Table V.

TABLE V.—RATES FOR TRANSFORMATION OF ANHYDROVITAMIN A INTO A VITAMIN A SUBSTANCE IN "95%" ISOPROPANOL

 $(k/2.303 \times \text{hr.}^{-1} \times 10^4)$ 

	"95%" Isopropanol Control	0.033 N Acetic Acid	0.033 N Caproic Acid	0.033 N Decanoic Acid	0.033 N Palmitic Acid
50°					
$k_1$	2.09	3.5	2.86	3.1	3.33
$k_2$	0.84	1.67	2.14	1.37	1.54
37°					
$k_1$	0.84	1.67	2.14	1.37	1.54
$k_2$	0.42	0.50	0.84	0.42	0.42

The addition of 5% water increased the reaction rates considerably (compare  $k_2$  of Table IV), thus enabling the rate at 25° to be measurable.

The rates of conversion in the "85%" isopropanol series are given in Table VI. Figure 5 represents a plot of the "85%" isopropanol control.

The rate appeared to vary directly with the increased water content and equilibrium was reached at one hundred and forty hours in the 50° samples. That the rates of the control, decanoic, and palmitic acid samples are almost equal could be explained by the fact that the latter two samples contained considerable amounts of oxidation products, giving the appearance of a slower rate of vitamin A substance formation.

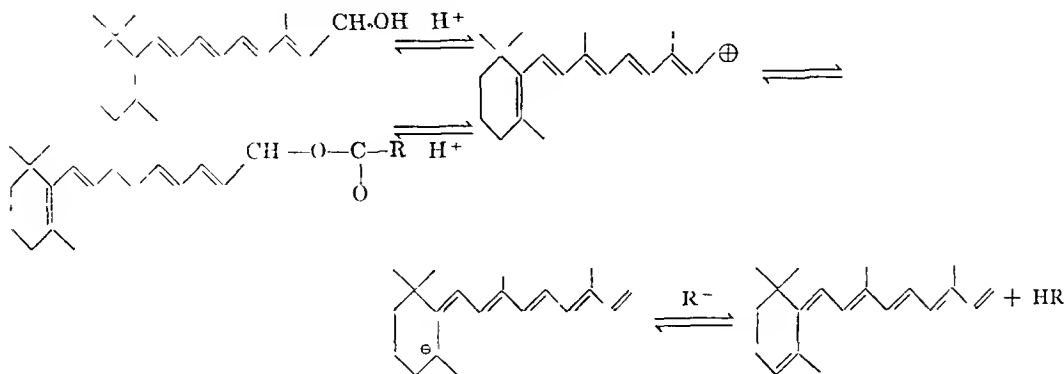
**Paper Chromatography.**—The isopropanol systems were chromatographed on paper using the method described under "95%" isopropanol-vitamin A acetate systems. All three isopropanol systems had the same qualitative picture. The controls had the following  $R_f$  values: vitamin A palmitate, 0.070; decanoate, 0.200; caproate, 0.526; acetate, 0.630; alcohol, 0.930; and anhydrovitamin A, 0.370. All the systems contained vitamin A alcohol, oxidation products, and anhydrovitamin A. The fatty acid solutions, in addition, contained an ester formed from the fatty acid of the media. It was also observed that the ratio of vitamin A alcohol to ester increased as the per cent of water of the media increased.

## DISCUSSION

**Effects of Fatty Acids.**—Caproic, decanoic, palmitic, stearic, and oleic acids demonstrated a stabilizing action on vitamin A esters in isopropanol when compared to a control without fatty acids. These data appeared to be different from the results obtained when vitamin A was dissolved in a vanishing cream base. These bases generally contain stearic acid, polyhydroxy alcohol, water, and an emulsifying agent, and the pH is usually on the basic side. Any one of these other ingredients could affect the destruction of vitamin A. The addition of 5% water to the isopropanol system increased the rate of elimination, but the fatty acid still retained some of its stabilizing action. The effects of water are in agreement with the results of Higuchi and Reinstein (1) who found that the rate of elimination of vitamin A acetate in hydroalcoholic media varied directly with the water content.

There were forms of vitamin A present in the isopropanol ester mixtures which were not originally



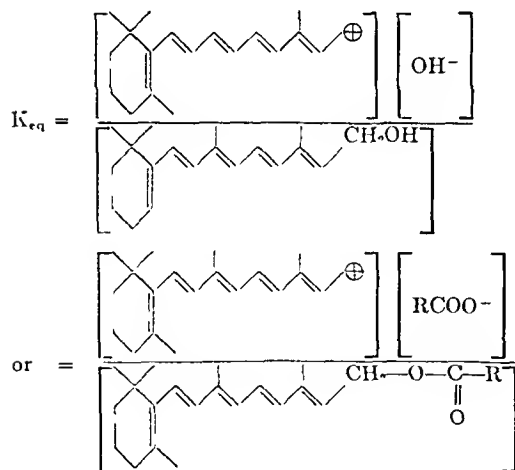


introduced as demonstrated by paper chromatography. This was believed to be due to a reaction of anhydrovitamin A with the fatty acid or water of the media.

**Action of Fatty Acids**—The control solutions without fatty acids invariably contained more anhydrovitamin A than the fatty acid solutions. Consequently, the increased stability of the esters in the presence of the fatty acids could be due to two reactions: (a) suppression of ionization of the ester, thus slowing down the rate of elimination, and (b) reaction of the fatty acid with anhydrovitamin A to reform a vitamin A substance.

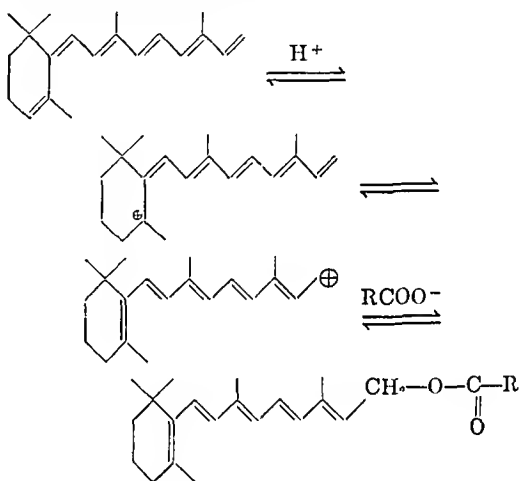
It has been theorized by Embree (10) that the initial step of vitamin A alcohol degradation in the presence of acids is the formation of the carbonium ion. It is also possible that this is the first step with vitamin A esters (see equation above).

This initial step could be considered as an ionization represented by a typical equilibrium equation



The effect of ( $RCOO^-$ ) or ( $OH^-$ ), the latter being known to prevent elimination of the acetate (1) can be seen from the law of mass action. Large quantities of these species can theoretically suppress ionization. The second suggested action of stabilization is possible since it is known that unsaturated compounds can undergo addition reactions with acids and also there are patents (7, 8) dealing with the conversion of anhydrovitamin A into vitamin A

active substances in acid media. This reaction could be represented as follows:



This possibility was investigated by studying anhydrovitamin A in cyclohexane, isopropanol "95%," and "85%" isopropanol solutions of fatty acids. The fatty acids and water reacted with the substrate to give vitamin A esters and alcohol, respectively, which was always faster in the presence of the fatty acid.

The chromatographic analysis of the anhydrovitamin A isopropanol systems showed that the "95%" isopropanol system contained vitamin A alcohol and ester as their active components, however, as the per cent of water increased, larger amounts of vitamin A alcohol were formed. Vitamin A treated with base will not eliminate (1), and the action of ( $H^+$ ) on vitamin A can be terminated by the addition of base (2). Similarly, anhydrovitamin A treated with base will not reform a vitamin A substance. It was found in this and other work (7, 8) that fatty acids catalyze the formation of a vitamin A substance from anhydrovitamin A. This information supports the second suggested reaction of stabilization and that this "formation" reaction is only acid catalyzed.

**Action of Water.**—Water had a catalytic effect in both the forward and reverse reaction (destruction and formation of vitamin A). This catalytic action could be related to increased ionization of the reactants. Other hydroxylated solvents as methanol,

TABLE VI.—RATES FOR TRANSFORMATION OF ANHYDROVITAMIN A INTO A VITAMIN A SUBSTANCE IN "85%" ISOPROPANOL

 $(k/2.303 \times \text{hr.}^{-1} \times 10^4)$ 

"85%" Isopropanol Control	0.033 N Acetic Acid	0.033 N Caproic Acid	0.033 N Decanoic Acid	0.033 N Palmitic Acid
$k$ (50°)	8.00	5.72	4.94	4.45
$k$ (37°)	6.45	4.45	4.43	4.00
$k$ (25°)	0.863	0.874	1.6	1.56

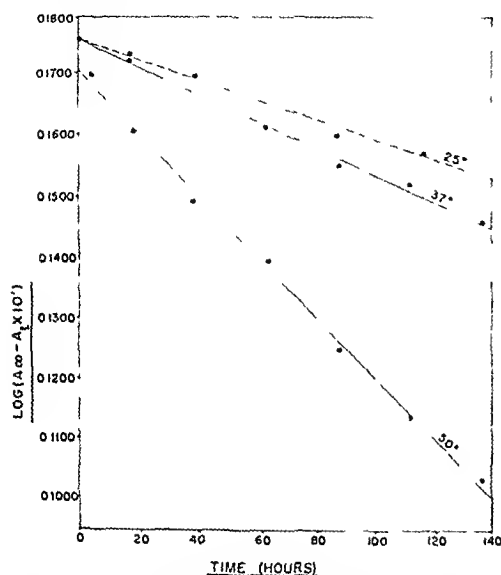


Fig. 5.—Anhydrovitamin A in "85%" isopropanol control.

ethanol, and isopropanol probably act by similar mechanisms but, due to lower polarity and solvating powers, they are less destructive.

Two rates,  $k_1$  and  $k_2$ , were found in the "99%" isopropanol series regardless of whether vitamin A ester or anhydrovitamin A was the substrate ( $k_1$ ,  $k_2$ ). It appeared that  $k_1$  and  $k_2$  were related to the small water content (0.056%) of the media, because 95% and 85% isopropanol series only demonstrated one rate.

It would be logical to assume that  $k_1$  is the rate due to water plus other factors. When the small amount of water was consumed, then  $k_2$ , which was slower, made its appearance. The fact that  $k_1$  was faster in both directions resembles the catalytic effect of water in the "95%" and "85%" systems, which tends to confirm the original idea.

## SUMMARY AND CONCLUSIONS

1. Vitamin A in hydrocarbon solvents as *n*-hexane solutions only showed oxidative degradation; in isopropanol, both oxidation and elimination had occurred.

2. The fatty acids in isopropanol solutions of vitamin A esters reduced the rate of vitamin A loss and maintained a lower concentration of anhydrovitamin A when compared to the control.

3. The addition of water to the isopropanol-vitamin A ester systems increased the rate of degradation and anhydrovitamin A formation. The fatty acids still maintained their stabilizing effect.

4. The reverse reactions, anhydrovitamin A plus isopropanol alone and with fatty acids showed that the fatty acid increased vitamin A formation when compared to a control.

5. Addition of water to systems mentioned in 4, increased rate of "vitamin A" substance formation.

6. In general, there does not appear to be any significant difference in the action of the different fatty acids studied.

7. The reaction of anhydrovitamin A plus fatty acid in cyclohexane was second order having a first-order dependence on each reactant.

8. The chromatographed fractions were identified by U. V. and I. R. spectra and  $R_f$  values.

9. The stabilizing action of the fatty acids appeared to be due to a regeneration of the ester in anhydrous solvents and a mixture of vitamin A alcohol and ester in hydroalcoholic media.

10. There appears to be a definite need for antioxidants in vitamin A solutions.

## REFERENCES

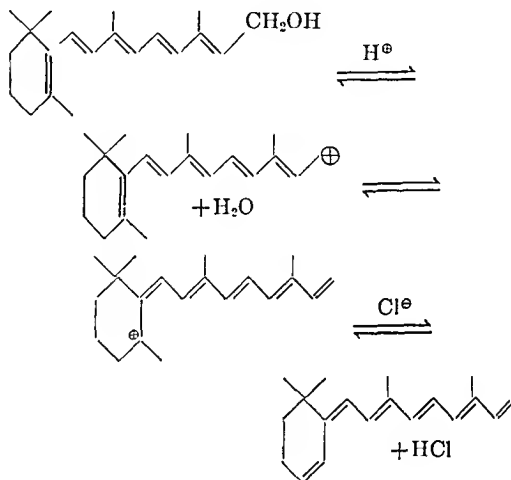
- (1) Higuchi, T., and Reinstein, J., *THIS JOURNAL*, 48, 155 (1959)
- (2) Shantz, E. J., *J. Am. Chem. Soc.*, 65, 901 (1943)
- (3) Chilcote, M. E., Gurreant, N. B., and Ellenberger, H. A., *Anal. Chem.*, 21, 960 (1949)
- (4) "United States Pharmacopoeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 942
- (5) "British Pharmacopoeia," The Pharmaceutical Press, London, England, 1953, p. 846
- (6) Cama, H., Collins, F. D., and Morton, R. A., *Biochem. J.*, 50, 48 (1952)
- (7) Embree, N., and Shantz, E., U. S. pat. 2,410,575, November 5, 1946
- (8) Orshnick, W., U. S. pat. 2,819,316, January 7, 1958
- (9) Frost, A., and Pearson, R., "Kinetics and Mechanisms," John Wiley & Sons, New York, N. Y., 1956.
- (10) Embree, N. J., *J. Biochem.*, 132, 619 (1940).

# Preparation and Stability of Some Esters of Vitamin A\*

By ALBERT J. FORLANO† and LOYD E. HARRIS

Vitamin A esters of fatty acids having electronegative groups in the number 2 position were prepared. Acids, with chlorine or a double bond, were used and the esters were tested for stability against acid and solvolytic action. The results indicate that (a) stability against proton attack, in an anhydrous solvent, varies directly with the  $K_a$  of the acid portion, (b) the addition of water to an anhydrous solvent, containing HCl, decreases the rate of formation of anhydrovitamin A, and (c) stability in alcoholic solvents, without an acid catalyst, varies inversely with the  $K_a$ . A combined mechanism of vitamin A ester degradation in the presence of mineral acids and hydrolytic solvents is presented.

THE ACTION of lower alcohols and strong acids upon vitamin A and its esters to produce anhydrovitamin A is a well established phenomenon Shantz, *et al* (1), refluxed vitamin A esters in ethanol and found that a mixture of anhydrovitamin A, fatty acid, vitamin A ethyl ether, and other products were formed and that the addition of HCl to the ethanol catalyzed this reaction. Vitamin A alcohol is more sensitive to elimination in acid solution than the esters; however, in the absence of the catalyst, vitamin A alcohol is more stable (2). Prolonged contact of anhydrovitamin A with ethanolic HCl produces a substance called isoanhydrovitamin A (1), which is formed by the addition of a molecule of solvent to the double bond in the cyclohexene ring Meunier (3) suggested a reasonable mechanism for ( $H^+$ ) catalysis of anhydrovitamin A formation from vitamin A alcohol. No information has been found regarding the mechanism of proton catalyzed decomposition of the ester; however, the mechanism shown below appears to be satisfactory.



Shantz, *et al*. (1), further substantiated the mechanism of ester decomposition when they found that anhydrovitamin A and fatty acid were formed when the ester was refluxed in ethanol. The greater stability of the ester in the presence of acid could be related to the electronegative effect of the carbonyl group on the alkyl oxygen. Reduction of the electron density around the alkyl oxygen should result in decreased attraction for protons, thus depressing the initial step of the reaction.

Based on this assumption, two series of esters were synthesized having electronegative groups in the 2 and 4 position of the fatty acid: (a) those containing chlorine in the  $\alpha$  position as vitamin A chloroacetate and  $\alpha$ -chloropropionate, and (b) those containing unsaturation in the 2 position as vitamin A acrylate, crotonate, and sorbate, the latter also having unsaturation in the 4 position.

Ingold (4) reported the  $K_a$ 's of the acids pertinent to this study. These are listed in Table I.

TABLE I— $K_a \times 10^5$  OF ACIDS

Acetic acid	1.75
Chloroacetic acid	155.00
Dichloroacetic acid	5100.00
Trichloroacetic acid	120,000.00
Propionic acid	1.34
$\alpha$ -Chloropropionic acid	147.00
Acrylic acid	5.56
Butyric acid	1.48
Crotonic acid	2.03
Sorbic acid	1.73

\* Received August 21, 1959, from the College of Pharmacy, The Ohio State University, Columbus 10.

Based, in part, upon a Ph.D. dissertation submitted to the Graduate School, The Ohio State University.

Presented to the Scientific Section, A.P.H.A., Cincinnati Meeting, August 1959.

† Present address: Chas. Pfizer & Co., Inc., Brooklyn, N.Y. The authors wish to express their gratitude to Mr. Paul Sleezer of Hoffmann-La Roche for his generous samples of vitamin A alcohol.

SYNTHESIS

**Materials Used in This Study.**—Vitamin A alcohol, Roche; chloroacetyl chloride, Eastman Organic (redistilled) b. p. 105–106°; trichloroacetyl chloride; Eastman Organic (redistilled) b. p. 114–116°;  $\alpha$ -chloropropionyl chloride, Eastman Organic (redistilled) b. p. 109–111°; pyridine dried over  $\text{CaCl}_2$  (redistilled) b. p. 114–115°; ethylene dichloride, Eastman Organic b. p. 82–84°; Woelm neutral activated alumina, Grade I for chromatography; petroleum ether b. p. 30–60°, Mallinckrodt (AR); quinoline, Eastman Organic b. p. 110–111°/14 mm.; N,N-dimethylaniline, Matheson, Coleman, and Bell b. p. 192–193.5°; sorbic acid, crotonyl chloride, and acrylyl chloride, Delta Chemical Co.

**General Method of Preparation of Esters.**—This method, a modification of the Baxter and Robeson method (5), was used for all the esters, except when modifications are specified.

Four and one-half grams (0.016 M) of vitamin A alcohol was dissolved in 25 ml. of ethylene dichloride containing 5 ml. of pyridine and cooled to 10° (I). In a separate flask, 0.018 M of the acid chloride was dissolved in 25 ml. ethylene dichloride (II). Solution II was added slowly to solution I with stirring and set in a dark place for two hours. The solvent was removed *in vacuo* and the residue was dissolved in 10 ml. of petroleum ether (30–60°). The instability of some of these esters necessitated their purification on alumina columns. The alumina was deactivated by the addition of 8% water in a glass-stoppered flask. After two hours of hydration, enough petroleum ether (30–60°) was added to the alumina to make a slurry which was poured into a glass column. Fifty grams of deactivated alumina were used for each Gm. of ester. The entire reaction mixture was poured on the column followed by petroleum ether (30–60°). Anhydrovitamin A was eluted first, followed by the ester. The former was distinguished from the latter by its orange color in U. V. light; the latter had a yellow-green color.

The ester fraction was evaporated *in vacuo* with the aid of a nitrogen bleeder, redissolved in petroleum ether, and rechromatographed as directed above. The residue was subjected to a high vacuum (0.5 mm.) for six hours at 35° to remove the pyridine or other bases. Quinoline and N,N-dimethylaniline were used in place of pyridine for the crotonate and acrylate esters, respectively. Attempts at crystallization from a variety of polar, nonpolar, and combination of both solvents were unsuccessful. The per cent yields are listed in Table II. Sorbic acid chloride was prepared by treating sorbic acid with thionyl chloride, *s. a.*, and collecting the fraction that distilled at 79–80° and 13 mm.

The saponification equivalents of these esters were determined potentiometrically by the Schriner and Fuson method (7). The results are tabulated in Table II.

**Infrared and U. V. Spectra.**—The infrared spectra of these compounds were determined on a Baird infrared spectrophotometer, using sodium chloride plates. The spectra showed definite ester carbonyl peaks and the absence of hydroxyl peaks. The positions of the carbonyl peaks are tabulated in Table III. The  $E(1\%, 1\text{cm.})$  values of the esters were determined in petroleum ether (30–60°) on a Beckman DU spectrophotometer at the U. V. maximum. The extinction coefficients and wavelength of the maxima are given in Table III.

TABLE III.—I. R. AND U. V. PHYSICAL CONSTANTS OF THESE ESTERS

Vitamin A Esters	Position Of	$E$ (1%, 1cm.) (U. V.)	U. V. Maximum ( $\mu$ )
	I. R. Peak ( $\mu$ )		
$\alpha$ -Chloropropionate	5.71	1300	328
Chloroacetate	5.64	1046 <sup>a</sup>	325
Sorbate	5.80	1318	327
Acrylate	5.74	1387	327
Crotonate	5.76	1318	327
Palmitate	5.73	963	327

<sup>a</sup> This low extinction coefficient is an indication of its lack of stability.

The complete U. V. spectra were determined on a Cary recording spectrophotometer in petroleum ether (30–60°). All the esters, except the chloroacetate, had typical vitamin A spectra (maxima 325–328  $\mu$ ). The chloroacetate showed impurity peaks at 310  $\mu$ , 341  $\mu$ , and 360  $\mu$ . Vitamin A sorbate had two peaks, one due to the chromophore of the vitamin A molecule, maximum at 327  $\mu$ , and the second due to the sorbate part at 255  $\mu$ .

**Determination of  $R_f$  Values and Purity.**—Using the system described in the previous paper (6), [6% liquid petrolatum in petroleum ether (30–60°)–ethanol, U. S. P.], the esters were chromatographed on paper as a means of identification and determination of purity. See Table IV.

The new esters with the exception of vitamin A chloroacetate, showed only one spot on the paper, indicating that they were pure. The chloroacetate appeared to be a mixture of anhydrovitamin A and ester, indicating the sensitivity of the ester to ethanol.

TABLE II.—PER CENT YIELD AND SAPONIFICATION EQUIVALENTS OF THESE ESTERS

Vitamin A Esters	% Yield	Molecular Weight	Saponification Equivalent	% Deviation from Theoretical
$\alpha$ -Chloropropionate	93	376	385	+2.61
Chloroacetate	82	362	329	−9.13 <sup>a</sup>
Trichloroacetate	b	...	...	...
Sorbate	80	381	385	+1.27
Crotonate	53	354	362	+2.21
Acrylate	45	340	347	+2.15

<sup>a</sup> The low saponification value is an indication of its poor stability.  
<sup>b</sup> This ester could not be made under these conditions.

# Preparation and Stability of Some Esters of Vitamin A\*

By ALBERT J. FORLANO† and LOYD E. HARRIS

Vitamin A esters of fatty acids having electronegative groups in the number 2 position were prepared. Acids, with chlorine or a double bond, were used and the esters were tested for stability against acid and solvolytic action. The results indicate that (a) stability against proton attack, in an anhydrous solvent, varies directly with the  $K_a$  of the acid portion, (b) the addition of water to an anhydrous solvent, containing HCl, decreases the rate of formation of anhydrovitamin A, and (c) stability in alcoholic solvents, without an acid catalyst, varies inversely with the  $K_a$ . A combined mechanism of vitamin A ester degradation in the presence of mineral acids and hydrolytic solvents is presented.

THE ACTION of lower alcohols and strong acids upon vitamin A and its esters to produce anhydrovitamin A is a well established phenomenon Shantz, *et al.* (1), refluxed vitamin A esters in ethanol and found that a mixture of anhydrovitamin A, fatty acid, vitamin A ethyl ether, and other products were formed and that the addition of HCl to the ethanol catalyzed this reaction. Vitamin A alcohol is more sensitive to elimination in acid solution than the esters, however, in the absence of the catalyst, vitamin A alcohol is more stable (2). Prolonged contact of anhydrovitamin A with ethanolic HCl produces a substance called isohanhydrovitamin A (1), which is formed by the addition of a molecule of solvent to the double bond in the cyclohexene ring. Meunier (3) suggested a reasonable mechanism for ( $H^+$ ) catalysis of anhydrovitamin A formation from vitamin A alcohol. No information has been found regarding the mechanism of proton catalyzed decomposition of the ester; however, the mechanism shown below appears to be satisfactory.

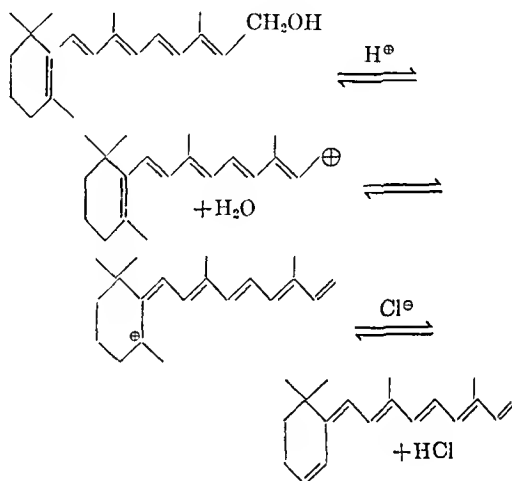


TABLE I— $K_a \times 10^3$  OF ACIDS

Acetic acid	1.75
Chloroacetic acid	155.00
Dichloroacetic acid	5100.00
Trichloroacetic acid	120,000.00
Propionic acid	1.34
$\alpha$ -Chloropropionic acid	147.00
Acrylic acid	5.56
Butyric acid	1.48
Crotonic acid	2.03
Sorbic acid	1.73

Shantz, *et al.* (1), further substantiated the mechanism of ester decomposition when they found that anhydrovitamin A and fatty acid were formed when the ester was refluxed in ethanol. The greater stability of the ester in the presence of acid could be related to the electronegative effect of the carbonyl group on the alkyl oxygen. Reduction of the electron density around the alkyl oxygen should result in decreased attraction for protons, thus depressing the initial step of the reaction.

Based on this assumption, two series of esters were synthesized having electronegative groups in the 2 and 4 position of the fatty acid: (a) those containing chlorine in the  $\alpha$  position as vitamin A chloroacetate and  $\alpha$ -chloropropionate, and (b) those containing unsaturation in the 2 position as vitamin A acrylate, crotonate, and sorbate, the latter also having unsaturation in the 4 position.

Ingold (4) reported the  $K_a$ 's of the acids pertinent to this study. These are listed in Table I.

\* Received August 21, 1959, from the College of Pharmacy, The Ohio State University, Columbus 10.

† Based, in part, upon a Ph.D. dissertation submitted to the Graduate School, The Ohio State University.

Presented to the Scientific Section, A.P.P.A., Cincinnati Meeting, August, 1959.

Present address, Chas. Pfizer & Co., Inc., Brooklyn, N.Y.  
The authors wish to express their gratitude to Mr. Paul Slezer of Hoffmann-La Roche for his generous samples of vitamin A alcohol.

## SYNTHESIS

**Materials Used in This Study.**—Vitamin A alcohol, Roche, chloroacetyl chloride, Eastman Organic (redistilled) b p 105–106°, trichloroacetyl chloride, Eastman Organic (redistilled) b p 114–116°,  $\alpha$  chloropropionyl chloride, Eastman Organic (redistilled) b p 109–111°, pyridine dried over  $\text{CaCl}_2$  (redistilled) b p 114–115°, ethylene dichloride, Eastman Organic b p 82–84°, Woelm neutral activated alumina, Grade 1 for chromatography, petroleum ether b p 30–60°, Mallinckrodt (AR), quinoline, Eastman Organic b p 110–111°/14 mm, *N,N*-dimethylaniline, Matieson, Colman, and Bell b p 192–193.5°, sorbic acid, erotonyl chloride, and acrylyl chloride, Delta Chemical Co

**General Method of Preparation of Esters**—This method, a modification of the Baxter and Robeson method (5), was used for all the esters, except when modifications are specified

Four and one half grams (0.016 M) of vitamin A alcohol was dissolved in 25 ml of ethylene dichloride containing 5 ml of pyridine and cooled to 10° (I). In a separate flask, 0.018 M of the acid chloride was dissolved in 25 ml ethylene dichloride (II). Solution II was added slowly to solution I with stirring and set in a dark place for two hours. The solvent was removed *in vacuo* and the residue was dissolved in 10 ml of petroleum ether (30–60°). The instability of some of these esters necessitated their purification on alumina columns. The alumina was deactivated by the addition of 8% water in a glass stoppered flask. After two hours of hydration, enough petroleum ether (30–60°) was added to the alumina to make a slurry which was poured into a glass column. Fifty grams of deactivated alumina were used for each Gm of ester. The entire reaction mixture was poured on the column followed by petroleum ether (30–60°). Anhydrovitamin A was eluted first, followed by the ester. The former was distinguished from the latter by its orange color in U V light, the latter had a yellow green color.

The ester fraction was evaporated *in vacuo* with the aid of a nitrogen bleeder, redissolved in petroleum ether, and rechromatographed as directed above. The residue was subjected to a high vacuum (0.5 mm) for six hours at 35° to remove the pyridine or other bases. Quinoline and *N,N*-dimethylaniline were used in place of pyridine for the crotonate and acrylate esters, respectively. Attempts at crystallization from a variety of polar, nonpolar, and combination of both solvents were unsuccessful. The per cent yields are listed in Table II. Sorbic acid chloride was prepared by treating sorbic acid with thionyl chloride, *s. a.*, and collecting the fraction that distilled at 79–80° and 13 mm.

The saponification equivalents of these esters were determined potentiometrically by the Schriner and Fison method (7). The results are tabulated in Table II.

**Infrared and U. V. Spectra.**—The infrared spectra of these compounds were determined on a Baird infrared spectrophotometer, using sodium chloride plates. The spectra showed definite ester carbonyl peaks and the absence of hydroxyl peaks. The positions of the carbonyl peaks are tabulated in Table III. The  $E(1\%, 1\text{cm})$  values of the esters were determined in petroleum ether (30–60°) on a Beckman DU spectrophotometer at the U V maximum. The extinction coefficients and wavelength of the maxima are given in Table III.

TABLE III — I R AND U V PHYSICAL CONSTANTS OF THESE ESTERS

Vitamin A Esters	Position Of I R Peak ( $\mu$ )	$E(1\% 1\text{cm})$ (U V)	U V Maximum ( $m\mu$ )
$\alpha$ Chloropropionate	5.71	1300	328
Chloroacetate	5.64	1046 <sup>a</sup>	325
Sorbate	5.80	1318	327
Acrylate	5.74	1387	327
Crotonate	5.76	1318	327
Palmitate	5.73	963	327

<sup>a</sup> This low extinction coefficient is an indication of its lack of stability.

The complete U V spectra were determined on a Cary recording spectrophotometer in petroleum ether (30–60°). All the esters, except the chloroacetate, had typical vitamin A spectra (maxima 325–328  $m\mu$ ). The chloroacetate showed impurity peaks at 310  $m\mu$ , 341  $m\mu$ , and 360  $m\mu$ . Vitamin A sorbate had two peaks, one due to the chromophore of the vitamin A molecule, maximum at 327  $m\mu$ , and the second due to the sorbate part at 255  $m\mu$ .

**Determination of  $R_f$  Values and Purity.**—Using the system described in the previous paper (6), [6% liquid petrolatum in petroleum ether (30–60°)-ethanol, U S P], the esters were chromatographed on paper as a means of identification and determination of purity. See Table IV.

The new esters with the exception of vitamin A chloroacetate, showed only one spot on the paper, indicating that they were pure. The chloroacetate appeared to be a mixture of anhydrovitamin A and ester, indicating the sensitivity of the ester to ethanol.

TABLE II — PER CENT YIELD AND SAPONIFICATION EQUIVALENTS OF THESE ESTERS

Vitamin A Esters	% Yield	Molecular Weight	Saponification Equivalent	% Deviation from Theoretical
$\alpha$ Chloropropionate	93	376	385	+2.61
Chloroacetate	82	362	329	-9.13 <sup>a</sup>
Trichloroacetate	<sup>b</sup>			
Sorbate	80	381	385	+1.27
Crotonate	53	354	362	+2.21
Acrylate	45	340	347	+2.15

<sup>a</sup> The low saponification value is an indication of its poor stability.

<sup>b</sup> This ester could not be made under these conditions.

TABLE IV.— $R_f$  VALUES OF VITAMIN A ESTERS AND RELATED COMPOUNDS

Compound	$R_f$ Value	Compound	$R_f$ Value
Neovitamin A alcohol	0 960	Vitamin A sorbate	0.570
Vitamin A alcohol	0 920	Vitamin A crotonate	0.550
Vitamin A methyl ether	0 725	Vitamin A chloroacetate	0.525
Vitamin A acetate	0 651	Vitamin A $\alpha$ -chloropropionate	0.515
Vitamin A acrylate	0 620	Vitamin A palmitate	0.100

**Stability of These Esters.**—The relative stability of these esters and related compounds toward eliminative degradation was determined in: (a) 0.01  $N$  HCl in anhydrous ethanol [water content 0.148% determined by Karl Fischer method (8)]; and (b) 0.01  $N$  HCl in ethanol U S P.

A sufficient quantity of material, such that the U V extinction at 326  $m\mu$  would be 0.700, was added to the acid solutions. These solutions were then transferred to silica cells and placed into a Beckman DU spectrophotometer and remained there through the entire determination. The temperature of the water flowing through the hydrogen lamp housing was maintained at  $15 \pm 1^\circ$ . Anhydrovitamin A has an extinction at 326  $m\mu$  equal to 0.2  $E_{326}$   $m\mu$ . The latter quantity was subtracted from the  $E_{326}$  observed to give a valid measurement of the vitamin A content at any time  $t$ . A plot of  $\log (E_{326} \text{ observed} - 0.2 E_{326})$  vs  $t$  was linear. Since all the ingredients except vitamin A were in a large excess the rate was calculated from the slope of the line. The rates are given in Table V.

TABLE V.—RATES OF ELIMINATION OF VITAMIN A SUBSTANCES IN 0.01  $N$  HCl IN ANHYDROUS ETHANOL<sup>a</sup> AND ETHANOL U S P

Vitamin A Substance	$\frac{k}{2.303} \times 10^4 \text{ min}^{-1}$	$\frac{k}{2.303} \times 10^4 \text{ min}^{-1}$
	(Anhydrous Ethanol)	(Ethanol U S P)
Vitamin A chloroacetate	b	b
Vitamin A alcohol	150.0	6.60
Neovitamin A alcohol	65.0	..
Vitamin A methyl ether	23.0	1.15
Vitamin A sorbate	15.0	0.70
Vitamin A acetate	12.5	1.10
Vitamin A palmitate	9.62	0.30
Vitamin A crotonate	9.35	0.60
Vitamin A acrylate	4.77	0.20
Vitamin A $\alpha$ -chloropropionate	2.32	6.82

<sup>a</sup> Water content 0.148%. <sup>b</sup> Too fast to measure.

The esters were tested for stability against eliminative degradation in isopropanol and decanoic acid in isopropanol. The solvents were "99%" isopropanol and "95%" isopropanol [containing 0.056% and 4.66% water, respectively, as determined by the Karl Fischer method (8)], and a second series containing 0.033  $N$  decanoic acid in both solvents. The solutions were prepared as described

in the previous paper (6) under sections dealing with vitamin A esters in isopropanol and "95%" isopropanol. They were assayed by the method of Cama, *et al.* (9). The starting concentrations of these solutions ranged from 6000–8000 u./ml. The initial 45° rates of decomposition of the various esters are given in Table VI according to the method previously described (6) by plotting log concentration vs time.

That the fatty acid did not appear to stabilize vitamin A chloroacetate in "95%" isopropanol is unexplainable at this time. The rates of the chloroacetate and  $\alpha$ -chloropropionate degradation at 45° and the other temperatures indicate their extreme sensitivity to solvolysis. The 37° and 45° sample of the chloro esters reached equilibrium in five hours, whereas the other esters required twenty to thirty hours to equilibrate.

**Chromatography of the Reaction Mixtures.**—The reaction mixtures from the isopropanolic solutions were paper chromatographed, using the method described previously (6) under the section dealing with vitamin A acetate in "95%" isopropanol. The identification of the zones was based on their  $R_f$  values previously described and U. V. spectra in ethanol U. S. P.; vitamin A esters maxima 325–328  $m\mu$ ; anhydrovitamin A 351, 371, and 391  $m\mu$ ; and oxidation products 275, 290, and 310  $m\mu$ .

All the samples contained the ester originally introduced, vitamin A alcohol, and oxidation products. The acrylate, crotonate, and sorbate samples with fatty acids also contained small quantities of vitamin A decanoate, and larger amounts of vitamin A alcohol than the controls. The samples of the chloro esters with the fatty acids did not appear to contain vitamin A decanoate.

## DISCUSSION

**Mechanism of Decomposition of Vitamin A Alcohol and Esters.**—The series of esters described in this paper was synthesized, based on the assumption that decreased electronegativity around the alkyl oxygen or carbonyl oxygen would decrease proton catalyzed attack.

When the stability of these esters was determined in "99%" and "95%" isopropanol, with and without a fatty acid present, their stability varied in this order:

Decreasing $K_a$ of Acid Portion of Ester	Vitamin A trichloroacetate Vitamin A chloroacetate Vitamin A $\alpha$ -chloropropionate Vitamin A acrylate Vitamin A crotonate Vitamin A sorbate Vitamin A acetate Vitamin A palmitate	Increasing Stability in Isopropanol
--	---	--

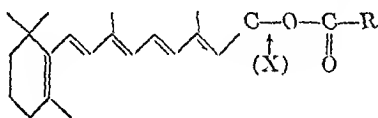
TABLE VI.—INITIAL DECOMPOSITION RATES OF VITAMIN A ESTERS IN ISOPROPANOLIC SOLUTIONS AT 45° ( $k/2.303 \times 10^3 \text{ hr.}^{-1}$ )

Ester	"99%" Isopropanol	"95%" Isopropanol	0.033 N Decanoic Acid in "99%" Isopropanol	0.033 N Decanoic Acid in "95%" Isopropanol
Acrylate	7.50	22.50	5.00	10.00
Chloroacetate	85.00	43.20	59.0	83.00
Sorbate	4.16	5.00	1.66	3.22
$\alpha$ -Chloropropionate	56.10	83.50	50.0	80.00
Crotonate	5.75	6.66	3.13	4.55
Palmitate <sup>a</sup>	1.37		1.25	
Acetate <sup>a</sup>		4.17	..	2.45

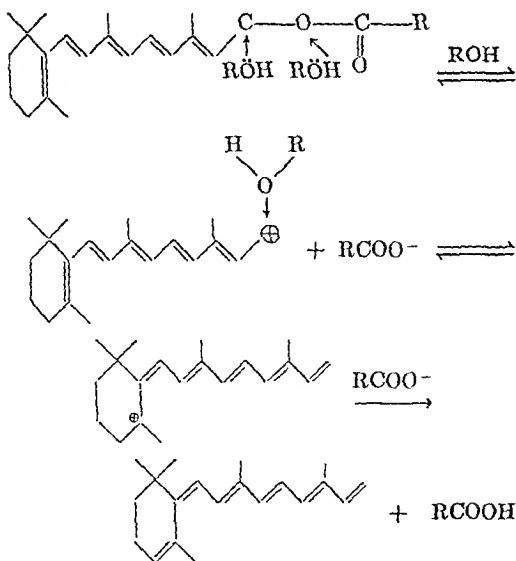
<sup>a</sup> Values derived from ref. (6)

Obviously there was a correlation between the  $K_a$  of the acid proton and decreased stability against elimination in isopropanol. The addition of 5% water to the anhydrous media catalyzed the elimination reaction which was to be expected.

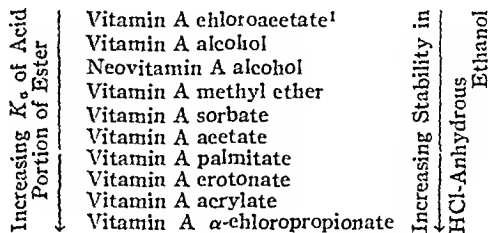
It appeared that the electronegative group in the 2 position increased the  $K_a$  of the acid by reduction of electron density around the alkyl oxygen; this factor also resulted in a weakening of the covalent alkyl oxygen bond (X). This weakening rendered it more susceptible to solvolysis in hydroalcoholic



and alcoholic media as shown by the stability tests. This was further substantiated by the fact that little or no anhydrovitamin A was formed in hydrocarbon solvents. The reaction could be represented as follows:

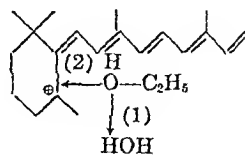


When the esters, vitamin A alcohol, neovitamin A alcohol, and methyl ether were treated with HCl in anhydrous ethanol, the stability was changed in this order:



The reason for the position of the chloroacetate can only be explained by the fact that the  $-\text{C}-\text{O}-$  bond is very sensitive to solvolysis in this compound, consequently, ethanol had sufficient solvolytic properties to destroy it rapidly. The stability of the other esters varied directly with the  $K_a$  of the acid radicals

The degradation rates of these compounds in HCl-ethanol U. S. P. did not follow any definite pattern. Their rates of decomposition appeared to be a composite of proton attack and solvolysis; the acrylate ester was the most stable. This addition of 5% water to the anhydrous media reduced the rate of elimination about 10 times. This appears to be related to a competition of water molecules and the proton on the cyclohexene ring for the oxygen of the ethanol, thus reducing the basicity of the media and rate of elimination of the proton.



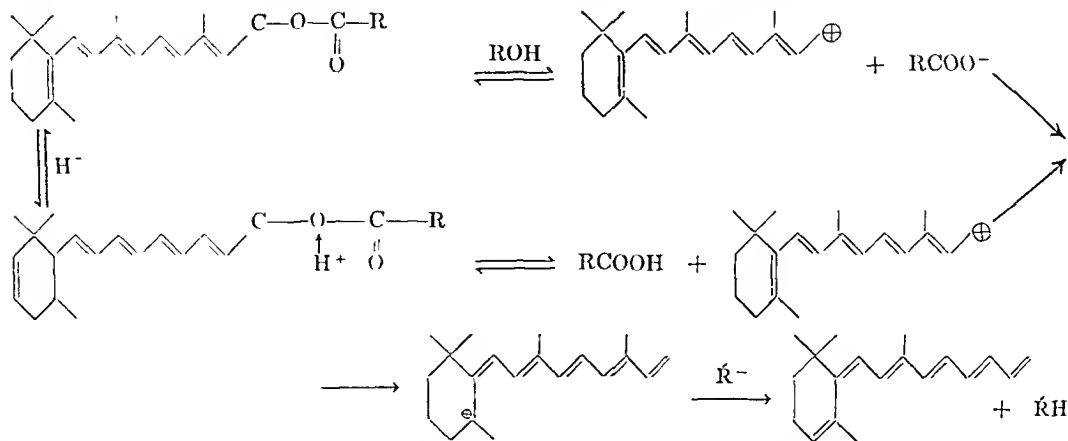
The differences in stability of these compounds in alcoholic solutions, with and without HCl suggested two mechanisms of decomposition.

(a) *Proton Catalyzed Attack.*—The esters containing fatty acids with large  $K_a$ 's were attacked the least and this appeared to be related to the reduced electron density around the alkyl oxygen. Since the electronegative effect of hydrogen is less than that of a carbonyl group it is not surprising that vitamin A alcohol is more sensitive to the proton catalyzed attack than vitamin A esters.

(b) *Solvolytic Attack.*—In alcoholic or hydroalco-

<sup>1</sup> This compound is an exception to the rule.





holic media the stability against elimination is dependent on the strength of the carbon-oxygen bond (X). It has been observed that vitamin A alcohol is more stable than vitamin A esters in uncatalyzed solvent systems (2). It is reasonable to assume that the electronegative effect of the carbonyl group and the groups in the  $\alpha$  position weakened the bond through an inductive effect on the electrons of the covalent bond. The electronegative effect of hydrogen (in vitamin A alcohol) is small compared to the strong electronegative groups mentioned. Solvolysis was further substantiated by Higuchi and Reinstein (2) who showed that pyridine in ethanolic vitamin A acetate solutions had no effect on the rate of elimination. Since pyridine would remove protons it appeared that the mechanism was solvolysis and not acid attack.

Stabilization by the one mechanism caused a corresponding decrease in stability by the other mechanism, therefore, it would appear most desirable to synthesize a molecule which is stable against solvolytic attack. This could possibly be done by the use of fatty acids with very low  $K_a$  values. The combined mechanism of decomposition is as shown above.

## SUMMARY AND CONCLUSIONS

1. A method of synthesis for esters of vitamin A sensitive to water was developed.

2. The rate of eliminative degradation of vitamin A esters in isopropanol, aqueous-isopropanol, and 0.01 N HCl in ethanol was measured.

3. The physical constants for these new esters were determined.

4. The previous information on vitamin A alcohol, its esters, and the information in this study was correlated into the possible mechanisms of vitamin A decomposition.

5. Paper chromatography was used to determine the reaction products.

## REFERENCES

- (1) Shantz, E. M., Cawley, J. D., and Embree, N. D., *J. Am. Chem. Soc.*, **65**, 901 (1943).
- (2) Higuchi, T., and Reinstein, J., *THIS JOURNAL*, **48**, 155 (1959).
- (3) Meunier, P., *Bull. soc. chim. biol.*, **25**, 371 (1943).
- (4) Ingold, C. K., "Structure and Mechanisms in Organic Chemistry," Cornell University Press, Ithaca, N. Y., 1953.
- (5) Baxter, J. G., and Robeson, C., *J. Am. Chem. Soc.*, **64**, 2407 (1942).
- (6) Forlano, A. J., and Harris, L. E., *THIS JOURNAL*, **49**, 451 (1960).
- (7) Schriner, R. L., and Fuson, C. R., "The Systematic Identification of Organic Compounds," John Wiley & Sons, New York, N. Y., 1956, p. 235.
- (8) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 941.
- (9) Cama, R., Collins, F. D., and Morton, R. A., *Biochem. J.*, **50**, 48 (1952).

# An Investigation of Particle-Medium Interactions in Suspensions\*

By W. A. HADDAD-LOUIS† and A. P. LEMBERGER

The physical significance of the two parameters as suggested by Robinson was investigated using Newtonian and non-Newtonian suspensions of glass spheres. System mean for each parameter did not vary significantly among the different suspensions. The initial viscosity and density of the medium seem to have no effect on the value of the parameters. An empirical equation used by Oliver can be rearranged when  $k_1 = 0$ , into a form similar to Robinson's equation. Since Robinson's equation can thus be viewed as a special case of the equation used by Oliver, it seems unlikely that the parameters have the physical significance assigned to them by Robinson.

RELATIVELY LITTLE is known about the influence of the dispersed phase on the overall rheological properties of suspensions, particularly at high volume concentrations of dispersed phase. Many empirical equations have been proposed which describe the rheological behavior of specific suspensions, but these equations cannot be made to apply to all systems.

Einstein derived a theoretically valid equation for the flow of simple systems under ideal conditions; however, the equation applies only at low volume concentrations of the dispersed phase. Several attempts have been made to extend Einstein's equation to higher volume concentrations of dispersed phase and to more complex systems. Some of these modified equations have been rather successful although they contain parameters without physical significance. Excellent reviews of the literature are given by Vand (1), Robinson (2, 3), and Ward (4).

Robinson (2, 3, 5) has proposed an equation with constants to which he has assigned physical significance. It was thought that equations which contain parameters to which physical significance can be attached could be usefully applied to a better understanding of the rheological properties of pharmaceutical suspensions and in a study of the contribution of the various components to the overall properties exhibited by a suspension. This study was undertaken to test the validity of the physical significance of the parameters in the equation proposed by Robinson.

## THEORETICAL CONSIDERATIONS

The equation derived by Einstein for the flow of simple systems under ideal conditions states

\*Received August 21, 1959, from the University of Wisconsin, School of Pharmacy, Madison.  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

This research project was supported in part by a grant from the Upjohn Co.

† Present address: Merck, Sharp and Dohme (I.A.) Corp., Cali, Colombia.

$$\eta = \eta_0(1 + KV) \quad (\text{Eq. 1})$$

where  $\eta$  is the viscosity of the suspension,  $\eta_0$  is the viscosity of the dispersing medium,  $V$  is the volume fraction per unit volume of suspension of dispersed phase, and  $K$  is a constant with a value of 2.5 for spheres (3).

Equation 1 may be rearranged into the form

$$\eta_{sp} = KV \quad (\text{Eq. 2})$$

where  $\eta_{sp}$ , the specific viscosity of the suspension, is equal to  $\eta/\eta_0 - 1$ .

In an attempt to extend the range of usefulness of Einstein's equation to higher volume concentrations, Robinson (2) proposed the equation

$$\eta_{sp} = KV/1 - S'V \quad (\text{Eq. 3})$$

where  $S'$  was defined as the relative sediment volume, the sediment volume per unit volume of solids. The quantity  $1 - S'V$  then corresponds to the volume of free liquid. Robinson tentatively assigned a physical significance similar to a frictional coefficient to  $K$ ; thus it would be influenced by such factors as a particle roughness, shape, and the presence or absence of an adsorbed shell of suspension medium around the particles. By plotting  $V/\eta_{sp}$  against  $V$ , the two parameters,  $K$  and  $S'$ , are given by the reciprocals of the  $V$  and  $X$  intercepts, respectively.

An empirical relation given by the equation

$$1 - \frac{1}{\eta_{rel}} = kV + k_1 \quad (\text{Eq. 4})$$

where  $\eta_{rel}$  is the relative viscosity ( $\eta/\eta_0$ ) and  $k$  and  $k_1$  are constants, was observed by Oliver (4, 6) to hold for suspensions. For stable suspensions  $k_1 = 0$ .

It can be simply shown that when  $k_1 = 0$ , Eq. 5 can be expressed as

$$\eta_{sp} = kV/1 - kV \quad (\text{Eq. 5})$$

which has the form of Robinson's equation. When  $k_1 \neq 0$ , Eq. 4 can be rearranged to

$$\eta_{sp} = \frac{kV + k_1}{1 - kV - k_1} \quad (\text{Eq. 6})$$

From Eqs. 5 and 6 it appears that when  $k_1 = 0$ , Robinson's  $K$  and  $S'$  should be equal to each other and identical with Oliver's  $k$ , while for suspensions where  $k_1 \neq 0$ , the values of  $K$  and  $S'$  should differ due to the introduction of the constant  $k_1$ .

## EXPERIMENTAL

**Apparatus.**—The Drage rheometer (7) was used for all the viscosity determinations in this study. The cup was immersed in a constant temperature bath set at  $25.0 \pm 0.1^\circ$  during the determinations.

**Materials.**—Glass spheres (Minnesota Mining and Manufacturing Co.) were used as suspended material in all experiments. The spheres obtained were subjected to a fractional sedimentation in water to eliminate hollow spheres. Microscopic analysis performed on the spheres used gave a mean diameter of  $30.08 \mu$ , with a standard deviation of  $3.94 \mu$ . A periodic check on the density of the spheres during the course of the study showed a change from 2.438 to 2.475, indicating that the size distribution remained essentially constant.

Solutions of commercially available grades of methyl cellulose (Dow Chemical Co.), carboxy methyl cellulose (Hercules Powder Co.), acaena U S P, sucrose U S P, and strontium bromide N F were used as suspending media. Polyethylene glycol 400 (Carbide and Carbon Chemicals Co.), S A E motor oils number 30 and 50 (Cities Service), and castor oil U S P were also employed as suspending media.

**Procedure.**—The required amount of glass spheres was weighed to the nearest 0.1 Gm and transferred to a calibrated Erlenmeyer flask. Some of the suspending medium was then added and mixed well with the glass spheres. Enough suspending medium was then added to dilute the suspensions to volume. The suspensions were then placed in a constant temperature water bath and allowed to come to temperature, about two hours was allowed.

Each suspension was then stirred well with a glass rod to break up any sediment which may have formed, the flask stoppered tightly, and allowed to tumble in a resuspending device for about ten minutes. The suspending medium was also treated in the same way. Enough of the suspension was then rapidly transferred to the proper cup and bob set, and the viscosity determined in the viscometer. The time elapsed between the transfer of the suspension to the cup and readings taken in the rheometer, both at increasing and decreasing rates of shear, was one to one and one half minutes. Several up and down determinations were made. A centering device consisting of two half circles, with an opening in the center for the neck of the bob, was used to make sure the bob was centered. All runs were repeated three to four times except for the strontium bromide solutions where single determinations were made. Suspensions of the glass spheres were prepared on a weight volume basis over a concentration range of 10–80% at 10% increments. Volume concentrations were calculated from the density of the glass spheres and suspension medium.

The glass spheres were recovered by repeated washings and decantations with distilled water. In some cases it was necessary to add a detergent to the water washings. In these cases, after washing, the glass spheres were allowed to stand overnight in chromic acid cleaning solution followed by repeated washing with distilled water until free of color. The spheres were then transferred to an evaporating dish and allowed to dry in an oven at  $105^\circ$  for ten hours.

## RESULTS AND DISCUSSION

**Calculation of Viscosities.**—It has been found empirically that a plot of the logarithm of the rate of shear vs the logarithm of the shearing stress yields a straight line, the slope of the line being dependent on the rheological behavior of the system. The equation of the straight line is

$$\log S = m \log \tau + \log a \quad (\text{Eq } 7)$$

which may be written

$$S = a\tau^m \quad (\text{Eq } 8)$$

where  $S$  is the rate of shear,  $\tau$  is the shearing stress,  $\log a$  is the intercept when  $\log \tau$  equals zero, and  $m$  is the slope of the line (8–15).

The apparent viscosity,  $\eta_a$ , of the system at any point on the curve is given by the derivative of  $\tau$  with respect to  $S$  therefore from equation 7

$$dS = am\tau^{(m-1)} d\tau \quad (\text{Eq } 9)$$

$$\frac{d\tau}{dS} = \frac{1}{ma\tau^{(m-1)}} \quad (\text{Eq } 10)$$

Equation 10 gives the viscosity of the system at any desired rate of shear, or shearing stress. When the substance behaves as a Newtonian liquid,  $m = 1$  and Eq 10 becomes

$$\frac{d\tau}{dS} = \frac{1}{a} \quad (\text{Eq } 11)$$

where  $1/a$  is the Newtonian viscosity. A convenient point to use as the basis of comparison for the viscosities of non-Newtonian substances is obtained by imposing the condition  $\tau = 1$ . Eq 10 then becomes

$$\frac{d\tau}{dS} = \frac{1}{ma} \quad (\text{Eq } 12)$$

The quantity  $1/ma$  is the apparent viscosity of the system when  $\tau = 1$ , and is the viscosity used in this investigation for non-Newtonian fluids.

The slope,  $m$ , is obtained from any two points of the curve. The constant,  $a$ , can be obtained, by calculation, from the slope and a point on the curve. Equation 12 has been used by several workers (8, 9, 14, 15) as a basis of comparison for the rheological behavior of non-Newtonian systems.

In order to obtain experimental data to support the usefulness of this viscosity measure a series of experiments was designed with solutions of methyl cellulose 25 and 4000 grades, which show pseudoplastic behavior. Solutions of increasing concentration of each grade of methyl cellulose with viscosities ranging from 50 to 500 cP were tested for their viscosities according to Eq 12 and the logarithms of the viscosities were plotted against the weight concentration of active ingredient. A straight line was obtained in each case. This behavior agrees with that obtained with other methods of viscosity determination.

**Systems Studied.**—Of the substances investigated, acaena and sucrose solutions, S A E motor oils 30 and 50, and polyethylene glycol 400 showed Newtonian behavior, while solutions of methyl cellulose 25 and 4000 grades, sodium CMC medium viscosity, and strontium bromide in methyl cellulose showed pseudoplastic behavior. The suspensions

of glass spheres in these substances behaved in the same way as did the original solutions of the substances.

Typical plots of Robinson's equation are shown in Figs 1 and 2. It should be pointed out that the viscosities at the low solid concentrations yield points which are less reliable than those at high solid concentrations, since in the calculation two large figures are being subtracted to obtain a small number. The equation held well in the range of concentrations used. The parameters obtained from Robinson's plots,  $K$  and  $S'$ , are summarized in Table I. It is to be noted that neither the viscosity nor the density of the original suspension seem to have any significant effect on the value

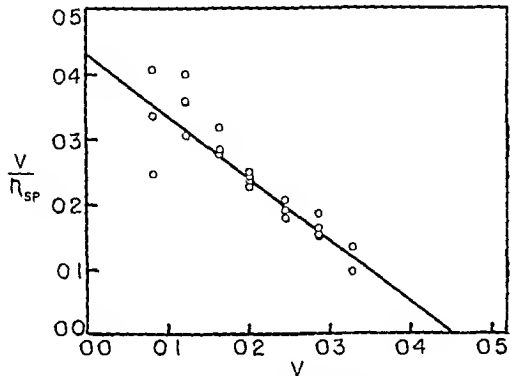


Fig. 1.—Robinson type plot of suspensions in CMC solution.  $K = 2.27$ ,  $S' = 2.17$ .

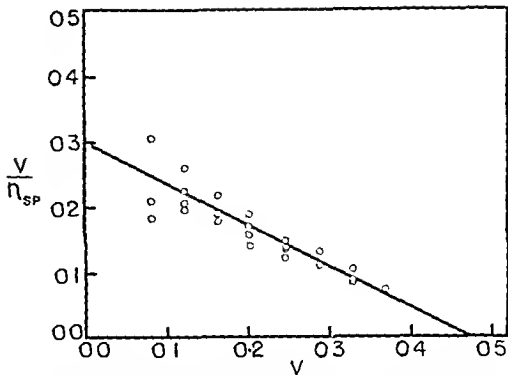


Fig. 2.—Robinson type plot of suspensions in methyl cellulose solution 4000 (initial viscosity = 227).  $K = 3.33$ ,  $S' = 2.11$ .

of  $K$ . Robinson reported similar results from his investigations with the exception of the  $K$  values, which he found to vary depending upon the medium and which he postulated as being influenced by particle-medium interaction.

The difference in diameters of the particles used in this work compared to the 10–30  $\mu$  spheres used by Robinson is to be noted. If there is assumed to be an immobile liquid shell of constant thickness around each particle, then the smaller the particle the greater the relative effect this shell would have on the effective particle volume. This may account for the difference in values obtained by Robinson and those presently reported.

TABLE I —PARAMETERS OBTAINED FROM ROBINSON'S PLOTS

Suspending Medium	Initial Viscosity, c p s	Density		$k$	$k_1$	Constants		$K$ (Calculated)
		Medium	Glass Spheres			$K$	$S'$	
Carboxy methyl cellulose (medium viscosity) soln.	188	1.0278	2.475	2.3	0.00	2.3	2.2	. .
Castor oil	710	0.9602	2.468	2.3	0.00	2.4	2.3	
Polyethylene glycol 400	88	1.1254	2.468	2.2	0.01	2.5	2.2	2.3
Sucrose solution	142	1.3270	2.468	2.3	0.01	2.7	2.3	2.4
Acacia solution	122	1.1246	2.470	2.0	0.11	3.0	2.3	2.8
Methyl cellulose 4000 soln.	133	1.004	2.438	2.0	0.10	2.8	2.1	2.9
Methyl cellulose 4000 soln.	227	1.004	2.438	2.0	0.15	3.3	2.1	3.5
Methyl cellulose 4000 soln.	507	1.004	2.438	2.1	0.07	3.0	2.1	2.8
Methyl cellulose 25 soln.	49	1.040	2.438	1.9	0.14	3.3	2.0	3.2
Methyl cellulose 25 soln.	220	1.040	2.438	1.9	0.09	3.2	1.8	2.9
Methyl cellulose 25 soln.	502	1.040	2.438	2.1	0.10	3.3	2.2	3.1
Strontium bromide in methyl cellulose 25 soln.	86	1.4818	2.468	1.8	0.15	3.2	1.9	3.2
Strontium bromide in methyl cellulose 25 soln.	108	1.5686	2.468	2.1	0.05	2.5	2.2	2.4
Strontium bromide in methyl cellulose 25 soln.	99	1.4545	2.468	2.0	0.08	2.7	2.1	2.6
Strontium bromide in methyl cellulose 25 soln.	98	1.2807	2.468	2.1	0.06	2.6	2.2	2.5
SAE No. 30 motor oil	211	0.8770	2.468	2.2	0.06	2.7	2.3	2.7
SAE No. 50 motor oil	555	0.8890	2.468	2.1	0.07	2.8	2.2	2.7

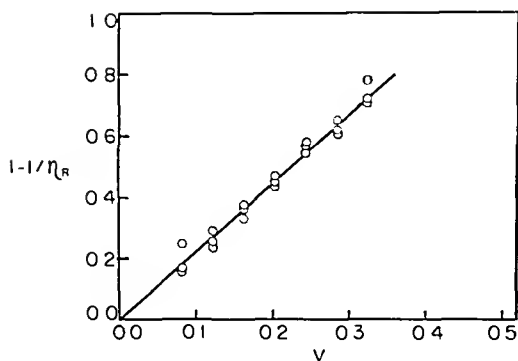


Fig. 3.—Oliver type plot of suspensions in CMC solution.  $k = 2.26$ ,  $k_1 = 0.00$ .

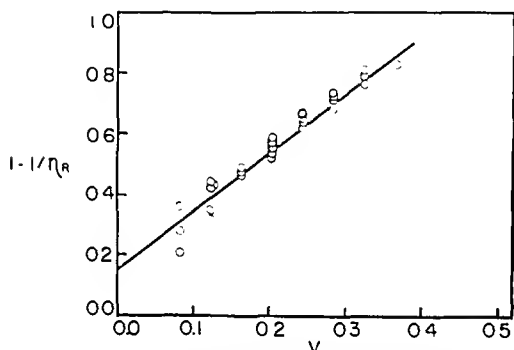


Fig. 4.—Oliver type plot of suspensions in methyl cellulose 4000 grade (initial viscosity = 227).  $k = 1.95$ ,  $k_1 = 0.15$ .

The value of  $S'$ , interpreted by Robinson as the relative sediment volume, showed a narrower variation range than did the values of  $K$ . The relative sediment volume per unit volume of glass spheres in water was obtained by multiplying the reciprocal of the bulk density by the true density of the glass spheres, thus:

$$1/1.41 \times 2.47 = 1.75$$

The bulk density was determined under the influence of gravity only. The difference in the values of the parameter  $S'$  determined experimentally and the relative packing volume of the glass spheres in water obtained here is significantly greater than that obtained by Robinson (2). If  $S'$  is the relative sediment volume, these results suggest that, in the media studied, a fairly thick layer of suspending medium is held immobile at the surface of the particles. This conclusion seems unlikely, however, in view of the small variation in  $S'$  observed with the different suspension media used in this study and the large size of the spheres used relative to molecular dimensions.

The data obtained in these studies have also been plotted according to the equation used by Oliver. Typical examples of these type plots are shown in Figs. 3 and 4. The values obtained for  $k$  and  $k_1$  from the slope and intercept, respectively, are given in Table I. In the cases where  $k_1 = 0$ , it can be observed that the values obtained for  $K$  and  $S'$  are close; they should in fact be equal. As  $k_1$  increases from zero it can be seen that  $K$  and  $S'$  differ more and more due to the incorporation of  $k_1$  into their values according to Eq. 6.

Since both Eqs. 3 and 6 seem to hold for the data obtained over the concentration range of suspended glass spheres used in this study, they may be equated.

$$n_{sp} = \frac{KV}{1 - S'V} = \frac{kV + k_1}{1 - kV - k_1}$$

and thus

$$K = \frac{(1 - S'V)(kV + k_1)}{V(1 - kV - k_1)} \quad (\text{Eq. 13})$$

If  $V$  is arbitrarily taken at some value at which both equations apply and the experimentally determined values for  $S'$ ,  $k$ , and  $k_1$  are used, it is possible to calculate the theoretical value of  $K$ . These calculated values are given in Table I where  $V$  is taken at 0.25.

The agreement obtained between the calculated and the experimental values for  $K$  seems reasonable in consideration of the possible error in  $S'$ ,  $k$ , and  $k_1$ , which are determined experimentally. It would thus seem that Robinson's equation can be viewed as a special case of the empirical equation used by Oliver. If the physical significance of  $K$  is as a frictional coefficient, then it must be corrected for some other factor which appears as  $k_1$  in Eq. 6. Indeed, since  $K$  and  $S'$  are expected to be numerically equal, it would seem that whatever physical significance they possess, if any, should be attributed to related properties.

## REFERENCES

- (1) Vand, V., *J. Phys. & Colloid Chem.*, **52**, 227(1958).
- (2) Robinson, J. V., *ibid.*, **53**, 1042(1949).
- (3) Robinson, J. V., *Trans. Faraday Soc.*, **1**, 15(1957).
- (4) Ward, S. G., *J. Oil & Colour Chemists' Assoc.*, **38**, 9(1955).
- (5) Robinson, J. V., *J. Phys. & Colloid Chem.*, **55**, 455(1951).
- (6) Eveson, G. F., *J. Oil and Colour Chemists' Assoc.*, **40**, 456(1957).
- (7) Operation Manual, Drage Products, Inc., 406 32nd St., Union City, N. J.
- (8) Maron, S. H., Madow, B. P., and Krieger, I. M., *J. Colloid Sci.*, **6**, 584(1951).
- (9) Farrow, F. D., Lowe, G. M., and Neale, S., *J. Textile Inst. Trans.*, **19**, 18(1928).
- (10) Porter, A. W., and Rau, P. A. M., *Trans. Faraday Soc.*, **23**, 311(1951).
- (11) Weltmann, R. N., N. A. C. A. Technical Note 3397, Washington, D. C., February, 1955.
- (12) Alfrey, T., "Mechanical Behavior of High Polymers," Interscience Publishers, New York, N. Y., 1918, p. 33.
- (13) Fischer, E. K., "Colloid Dispersions," John Wiley & Sons, New York, N. Y., 1950, p. 168.
- (14) Merrill, E. W., *J. Colloid Sci.*, **11**, 1(1956).
- (15) Krieger, I. M., and Maron, S. H., *ibid.*, **6**, 528(1951).

# Accelerated Color Loss of Certified Dyes in the Presence of Nonionic Surfactants\*

By MORTON W. SCOTT†, ALEX J. GOUDIE, and ARTHUR J. HUETTEMAN

The retention of color in aqueous and buffered solutions containing certified dyes (0.001 per cent) and nonionic surfactants (1.0 per cent) was measured after fourteen days storage at 48.8°. Six dyes, (FD&C Red No. 1, Blue No. 2, Red No. 4, Green No. 1, Yellow No. 5, and D&C Orange No. 3) and five commercially available surfactants (Tween 20, Igepal CA-710, Pluronic F-68, Myrj 52, and Brij 35) were included in the study. In all but four of the thirty systems examined, accelerated fading was noted over that obtained with solutions of the dye alone. For mixtures of FD&C Red No. 1 with Brij 35, and mixtures of FD&C Blue No. 2 with Pluronic F-68, the rate of fading was found to follow pseudo first order kinetics.

CONSIDERABLE EFFORT is often required in formulating color-stable pharmaceuticals containing certified dyes. While the use of antioxidants, chelating agents, and/or light absorbers may be of value in specific formulations, these additives do not exert protective effects in all systems. In the past, the formulating chemist also had at his disposal a reasonable number of certified dyes within each color category so that it was generally possible to select, after simple screening studies, at least one dye possessing satisfactory stability characteristics. Since the number of dyes on the certified lists now seems to be decreasing annually, the selection of a suitable dye is becoming more difficult.

Although the problem of maintaining color stability in pharmaceuticals and cosmetics is of general concern, little pertinent information on this problem has appeared in the literature. It is significant to note that quantitative data on the light and thermal stability of the certified dyes is not readily available in published reports. Some information can be obtained from general reviews released by the various dye manufacturers (1-3), but this data is only qualitative in nature. Details on the spectral characteristics and manufacturing techniques for several certified dyes can be found in the reports of Sclar and Freeman (4, 5). However, the interactions between dyes and other pharmaceutical reagents which may lead to poor color stability in finished products have received little attention.

Kuramoto, Lachman, and Cooper (6) have shown recently that reducing sugars, such as dextrose and lactose, have a deleterious effect on the color stability of FD&C Blue No. 2. While these results are not surprising in view of the known sensitivity of indigo dyes to reducing agents (7), the report does illustrate how common

pharmaceutical materials may react with dyes to promote fading.

The present study was prompted by observations in our laboratories which suggested that nonionic surfactants may accelerate the rate of fading in products containing certified dyes. Since these surfactants are often used in colored preparations, it appeared worthwhile to explore this area in greater detail.

To simplify our experiments, it was decided to examine the color stability of dye-surfactant mixtures using simple aqueous solutions of the two components. For the initial survey experiments, six dyes, representing each of the major color categories, and five nonionic surfactants were selected. The surfactants included both ester and ether types which are widely used in pharmaceuticals. The dyes and surfactants were studied at 0.001% and 1.0%, respectively, because these compounds are commonly employed in similar concentrations in many formulations.

The systems containing FD&C Red No. 1 with Brij 35 and FD&C Blue No. 2 with Pluronic F-68 were taken for more detailed kinetic analysis. These systems were selected because they represented dye-surfactant combinations with widely differing properties and because their rates of color loss seemed most amenable to kinetic study.

## EXPERIMENTAL

**Materials.**—Certified dyes used in this study included: FD&C Red No. 1, FD&C Blue No. 2, FD&C Red No. 4, FD&C Green No. 1, FD&C Yellow No. 5, and D&C Orange No. 3.<sup>1</sup> The commercially available nonionic surfactants employed for the study were: Tween 20,<sup>2</sup> Igepal CA-710,<sup>3</sup> Pluronic F-68,<sup>4</sup> Myrj 52,<sup>5</sup> and Brij 35.<sup>6</sup>

<sup>1</sup> Dyes obtained from H. Kohnstamm Co., Inc.

<sup>2</sup> Polyoxyethylene sorbitan monolaurate, Atlas Powder Co.

<sup>3</sup> Alkyl phenoxy polyoxyethylene ethanol, Antara Chemicals Division, General Dyestuff Corp.

<sup>4</sup> Condensate of polyoxypropylene with ethylene oxide, Wyandotte Chemicals Corp.

<sup>5</sup> Polyoxyethylene stearate, Atlas Powder Co.

<sup>6</sup> Polyoxyethylene lauryl ether, Atlas Powder Co.

\* Received August 21, 1959, from Johnson and Johnson Research, New Brunswick, N. J.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: Revlon, Inc., Bronx, N. Y.

TABLE I.—COLORIMETRIC DATA FOR DYE-SURFACTANT MIXTURES IN AQUEOUS SOLUTION

Dye-Surfactant Combinations <sup>a</sup>	Initial Observations		Observations After 14 Days at 48.8°	
	Absorbance <sup>b</sup>	Ratio of Sample to Control Absorbances	Absorbance <sup>b</sup>	Ratio of Sample to Control Absorbances
FD&C Red No. 1				
None	0.492	...	0.479	...
Tween 20	0.425	0.866	c	...
Igepal CA-710	0.430	0.875	0.287	0.600
Pluronic F-68	0.482	0.983	0.470	0.983
Myrj 52	0.443	0.902	0.000	0.000
Brij 35	0.439	0.893	0.094	0.196
FD&C Blue No. 2				
None	0.451	...	0.243	...
Tween 20	0.290	0.643	c	...
Igepal CA-710	0.415	0.922	0.000	0.000
Pluronic F-68	0.455	1.010	0.061	0.251
Myrj 52	0.430	0.955	0.007	0.003
Brij 35	0.305	0.698	0.000	0.000
FD&C Red No. 4				
None	0.452	...	0.420	...
Tween 20	0.388	0.858	c	...
Igepal CA-710	0.387	0.855	0.293	0.710
Pluronic F-68	0.437	0.968	0.403	0.960
Myrj 52	0.404	0.893	0.009	0.021
Brij 35	0.396	0.876	0.060	0.143
FD&C Green No. 1				
None	1.495	...	1.321	...
Tween 20	0.381	0.393	c	...
Igepal CA-710	1.031	0.690	0.140	0.095
Pluronic F-68	1.372	0.918	1.249	0.942
Myrj 52	1.459	0.975	0.019	0.007
Brij 35	1.495	1.000	0.052	0.025
FD&C Yellow No. 5				
None	0.504	...	0.491	...
Tween 20	0.504	1.000	c	...
Igepal CA-710	0.503	1.000	0.439	0.934
Pluronic F-68	0.499	0.992	0.080	0.163
Myrj 52	0.492	0.980	0.000	0.000
Brij 35	0.489	0.972	0.002	0.004
D&C Orange No. 3				
None	0.443	...	0.281	...
Tween 20	0.430	0.972	c	...
Igepal CA-710	0.421	0.952	0.157	0.558
Pluronic F-68	0.445	1.005	c	...
Myrj 52	0.438	0.990	0.049	0.174
Brij 35	0.433	0.978	0.159	0.566

<sup>a</sup> All dyes used at concentrations of 0.001% w/v; surfactant concentration at 1.0% w/v.<sup>b</sup> Absorbance measured at wavelength of maximum absorption: FD&C Red No. 1 at 504 mμ, FD&C Blue No. 2 at 609 mμ, FD&C Red No. 4 at 503 mμ, FD&C Green No. 1 at 627 mμ, FD&C Yellow No. 5 at 413 mμ, D&C Orange No. 3 at 480 mμ.<sup>c</sup> Sample was colorless and turbid.

**Preparation of Samples for Survey.**—Aqueous stock solutions of each dye and each surfactant were prepared at concentrations of 0.01% and 10.0%, respectively. In the case of the Pluronic F-68, Myrj 52, and Brij 35, mild heat and agitation were needed to speed dissolution of the surfactant. Aliquots of the stock solutions were blended and diluted with distilled water to give a series of 30 solutions, each containing a single dye at 0.001% and one surfactant at 1.0%. All possible 2-component combinations of the six dyes and five surfactants were prepared.

Samples were packaged in 50-ml. volumetric flasks (filled to the mark) and stored in the dark at 48.8 ± 0.5° for fourteen days. They were cooled quickly to room temperature, brought back to volume with distilled water if necessary, and analyzed. Control samples containing no surfactant and sample blanks containing no dye were handled in a similar fashion. A duplicate series of experiments was conducted in which M/15 Soren-

sen's phosphate buffer, pH 6.98, was used as the diluent for all stock solutions and samples.

**Analysis.**—The visible absorption spectra for the initial and aged samples were obtained with a Beckman model DK-1 recording spectrophotometer using 1.0-cm. Pyrex cells. pH values were determined with a Beckman Zeromatic pH meter.

**Kinetic Studies.**—Solutions containing 0.001% FD&C Red No. 1 combined with 1.0% Brij 35 and 0.001% FD&C Blue No. 2 combined with 1.0% Pluronic F-68 in M/15 Sorenson's phosphate buffer, pH 6.98, were prepared as outlined above. Aliquots of these solutions were stored at 21.1, 37.5, 48.8, 60.0, and 71.1 ± 0.5° for various time intervals. Control samples containing no surfactant were stored under the same conditions. At appropriate times, samples were removed from each temperature station for colorimetric analysis. A Beckman model DU spectrophotometer was used for all measurements. The samples were scanned occasionally on the model DK-1 instrument to

TABLE II.—COLORIMETRIC DATA FOR DYE-SURFACTANT MIXTURES IN M/15 SORENSSEN'S PHOSPHATE BUFFER, pH 6.98<sup>a</sup>

Dye-Surfactant Combinations <sup>b</sup>	Initial Observations		Observations After 14 Days at 48.8°	
	Absorbance <sup>c</sup>	Ratio of Sample to Control Absorbances	Absorbance <sup>c</sup>	Ratio of Sample to Control Absorbances
FD&C Red No. 1				
None	0.444	...	0.441	...
Tween 20	0.391	0.881	0.003	0.007
Igepal CA-710	0.382	0.862	0.024	0.054
Pluronic F-68	0.430	0.970	0.410	0.930
Myrj 52	0.392	0.884	0.000	0.000
Brij 35	0.387	0.895	0.002	0.005
FD&C Blue No. 2				
None	0.410	...	0.333	...
Tween 20	0.410	1.000	0.139	0.416
Igepal CA-710	0.409	0.997	0.291	0.874
Pluronic F-68	0.414	1.008	0.226	0.679
Myrj 52	0.404	0.985	0.143	0.430
Brij 35	0.380	0.927	0.000	0.000
FD&C Red No. 4				
None	0.477	...	0.479	...
Tween 20	0.414	0.869	0.002	0.004
Igepal CA-710	0.399	0.837	0.198	0.413
Pluronic F-68	0.460	0.965	0.457	0.955
Myrj 52	0.407	0.853	<sup>d</sup>	...
Brij 35	0.400	0.840	0.000	0.000
FD&C Green No. 1				
None	0.999	...	0.978	...
Tween 20	0.057	0.057	0.058	0.057
Igepal CA-710	0.085	0.085	0.081	0.079
Pluronic F-68	0.843	0.844	0.776	0.823
Myrj 52	0.040	0.040	0.040	0.039
Brij 35	0.046	0.046	0.032	0.031
FD&C Yellow No. 5				
None	0.487	...	0.475	...
Tween 20	0.487	1.000	0.008	0.017
Igepal CA-710	0.477	0.979	0.214	0.451
Pluronic F-68	0.487	1.000	0.467	0.983
Myrj 52	0.473	0.971	0.008	0.017
Brij 35	0.479	0.982	0.002	0.004
D&C Orange No. 3				
None	0.410	...	0.407	...
Tween 20	0.393	0.958	0.000	0.000
Igepal CA-710	0.380	0.926	0.202	0.496
Pluronic F-68	0.403	0.985	0.396	0.973
Myrj 52	0.398	0.970	0.000	0.000
Brij 35	0.381	0.930	0.000	0.000

<sup>a</sup> All samples remained at pH 6.98 ± 0.02 over the storage interval.<sup>b</sup> All dyes used at concentrations of 0.001% w/v; surfactant concentration at 1.0% w/v.<sup>c</sup> Absorbance measured at wavelength of maximum absorption; FD&C Red No. 1 at 504 mμ, FD&C Blue No. 2 at 609 mμ, FD&C Red No. 4 at 503 mμ, FD&C Green No. 1 at 627 mμ, FD&C Yellow No. 5 at 413 mμ, D&C Orange No. 3 at 480 mμ.<sup>d</sup> Sample was colorless and turbid.

determine if their wavelengths of maximum absorbance had shifted. In no case was such a shift detected. pH values were found to remain constant over the test interval.

## RESULTS AND DISCUSSION

Table I summarizes the absorbance data for all unbuffered dye-surfactant combinations included in this study. The intensity of color remaining in the dye-surfactant samples compared to that remaining in the surfactant-free controls is also shown in Table I for both the initial and aged samples. This comparative retention of color has been expressed in terms of the ratio of the sample absorbance to that of the control, where both measurements were taken at the wavelength of maximum absorbance for the dye component alone. The selection of this wavelength for comparison

was justified since it was found that in the presence of surfactant no wavelength shift occurred. Absorbance measurements were taken for all FD&C Red No. 1 systems at 504 mμ, Blue No. 2 at 609 mμ, Red No. 4 at 503 mμ, Green No. 1 at 627 mμ, and D&C Orange No. 3 systems at 480 mμ.

The results in Table I show that each of the surfactants tested exerts a deleterious effect on the color stability of the six certified dyes. After fourteen days aging at 48.8°, 26 of the 30 systems examined exhibited a comparative color loss greater than 10%. In general, FD&C Blue No. 2 appeared most sensitive to the action of the surfactants, while Tween 20 and Myrj 52 produced the most dramatic effects on dye stability. In the systems containing Tween 20 and FD&C Blue No. 2 or Red No. 4, significant fading was noted immediately after preparation of the samples.



In all cases reported in Table I, a significant drop in sample pH from the initial values between 6.5-7.0 was obtained after aging. The final pH fell in the range of pH 3.5 to 5.0, although there was considerable variation between each surfactant system. Similar observations were obtained for the surfactant solutions containing no dye. In the case of the ester type surfactants (Tween 20 and Myrj 52) this pH drop is believed to be the result of hydrolysis. This may, in turn, explain the appearance of turbidity in several of the Tween 20 samples. For the ether surfactants, the pH drop on aging is more difficult to explain. No indications of microbiological contamination were found in any of the fourteen day samples.

In order to eliminate the possibility that dye fading was associated with the increase in acidity in aged samples, all dye surfactant combinations were reprepared in buffer solution. The results of this study are shown in Table II. It is apparent that, while the pH has remained constant over the storage interval, accelerated fading still occurs. We have deliberately avoided making a comparison of the fading effects in the buffered and unbuffered solutions because different lots of surfactants were used for each study.

The exact interactions between dye and surfactant which lead to accelerated fading are not known. It would appear from the results in Tables I and II that some characteristic common to all of the surfactants is involved in the fading mechanism. Since all of the surfactants studied are ethylene oxide addition products it seemed worthwhile to examine the effects of other ethylene oxide condensates on dye stability. Limited experiments using polyethylene glycol 200, polyethylene glycol 600, and polyethylene glycol 6000, showed that these materials also cause accelerated fading of the certified dyes. The reaction of dyes with surfactants may result therefore because of the presence of the ethylene oxide moiety in the surfactants. While we do not postulate a complexing phenomenon at this time, Waibel (5) has reported that (ethoxylated) nonionic surfactants exist in solution as polyoxonium compounds which actually are not nonionic, but weakly cationic. He states that these positively charged ions are capable of reacting with anionic coloring agents.

Figure 1 shows that the rate of fading of FD&C Red No. 1 in the presence of Brij 35 proceeds by two pseudo first order reactions. The first reaction, which is comparatively slow, was observed at all temperatures. This was followed at temperatures above 48.8° by a more rapid second reaction. Table III presents the rate constants at the five temperatures studied.

It is important to note further that FD&C Red No. 1 in the absence of Brij 35 did not fade at any of the temperatures investigated. The accelerating effects of the surfactant, therefore, are clearly evident.

The Arrhenius plots for the Red Brij system are shown in Figs. 2 and 3. These plots suggest that two mechanisms are involved in the fading reaction. At temperatures below 37.5° we find the first reaction which is characterized by an energy of

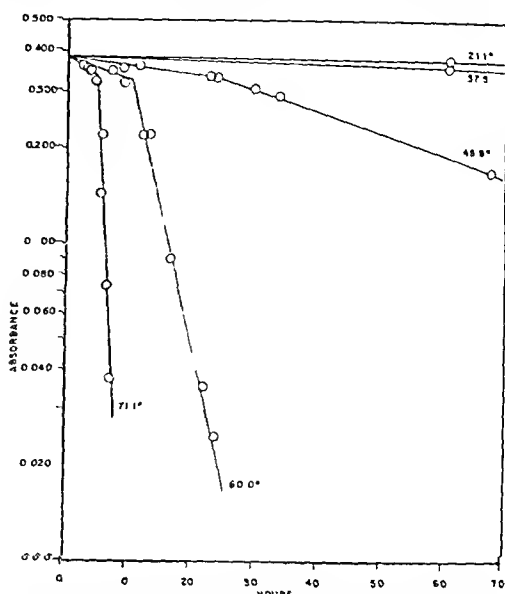


Fig 1—First order plots for color fading in FD&C Red No. 1-Brij 35 system

TABLE III—RATE CONSTANTS FOR THE FADING OF COLOR IN FD&C RED NO. 1-BRIJ 35 MIXTURES AT VARIOUS TEMPERATURES

Composition	Temperature °C ±0.5° C	Rate Constants (Reciprocal Hours)	
FD&C Red No. 1 (0.001%)	21.1	$<1.0 \times 10^{-8}$	
	37.5	$<1.0 \times 10^{-6}$	
	48.8	$<1.0 \times 10^{-5}$	
	60.0	$<1.0 \times 10^{-3}$	
	71.1	$<1.0 \times 10^{-3}$	
FD&C Red No. 1 (0.001%) plus Brij 35 (1.0%)	21.0	First Reaction	Second Reaction
	37.5	$1.64 \times 10^{-4}$	$1.25 \times 10^{-3}$
	48.8	$5.94 \times 10^{-3}$	$1.53 \times 10^{-3}$
	60.0	$1.51 \times 10^{-2}$	$1.85 \times 10^{-1}$
	71.1	$3.89 \times 10^{-2}$	$8.09 \times 10^{-1}$

\* Diluent: Sorensen's M/15 phosphate buffer, pH 6.98

activation of 21.9 Kcal/mole and a probability factor of  $3.8 \times 10^{-11}$ . At higher temperatures the second reaction occurs which has an energy of activation of 47.0 Kcal/mole and a probability factor of  $1 \times 10^{10}$ . These values are viewed with some reservation because replicate experiments showed that the kinetics may be altered significantly by what we believe to be lot to lot variations in the surfactant.

Figures 4 and 5 show that the rate of fading of FD&C Blue No. 2 in the absence and presence of Pluronic F 68 also follows pseudo first order kinetics. In this system, no induction period was obtained although Kuramoto, *et al.*, have previously reported its presence (6). Table IV summarizes the rate data and Arrhenius plots for these systems are presented in Fig. 6.

It is apparent that in this particular study Pluronic F 68 produces a negligible effect on the color stability of the blue dye. In replicate studies

\* At the lower temperatures observations were extended considerably beyond that shown in Fig. 1.

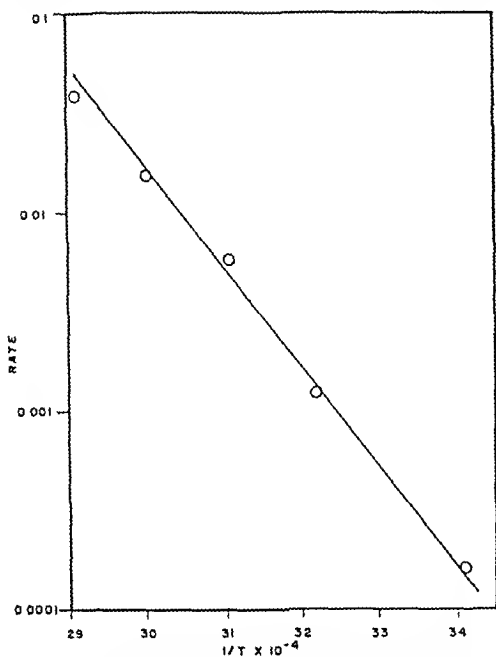


Fig. 2.—Arrhenius plot for first fading reaction in FD&C Red No. 1-Brij 35 system

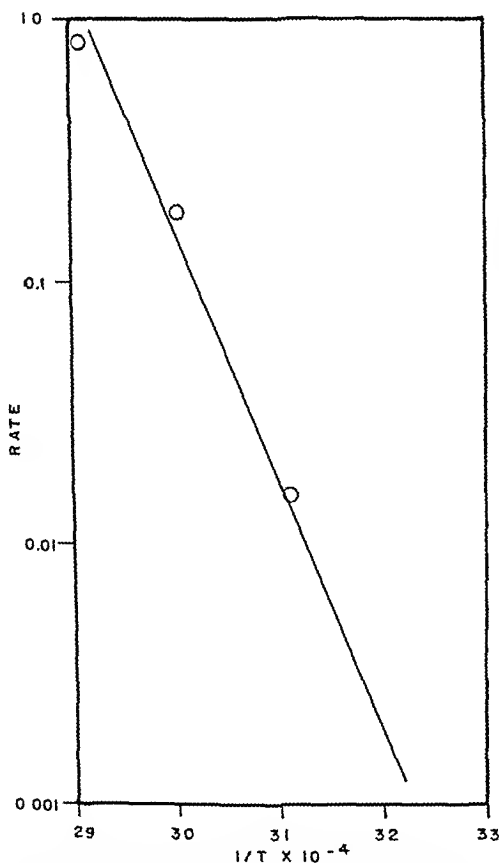


Fig. 3.—Arrhenius plot for second fading reaction in FD&C Red No. 1-Brij 35 system.

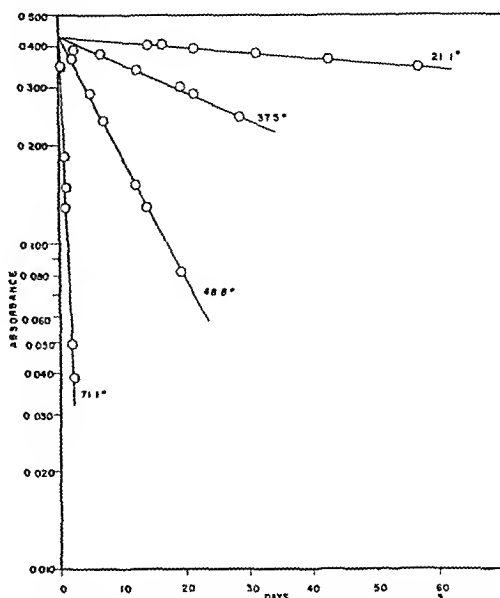


Fig. 4.—First order plots for color fading of FD&C Blue No. 2.

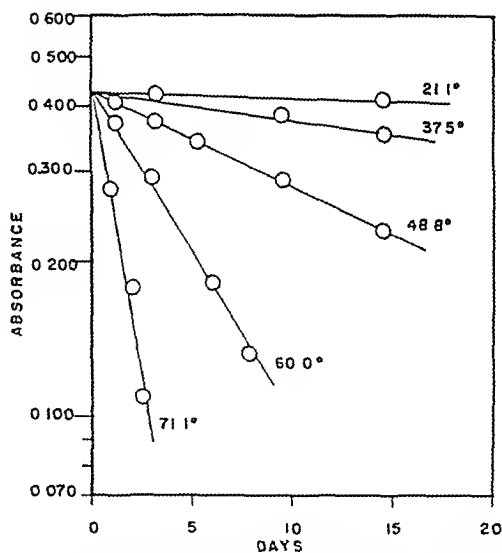


Fig. 5.—First order plots for color fading in FD&C Blue No. 2-Pluronic F-68 system.

we again noted that different kinetics may be obtained. For example, in one study the presence of Pluronic F-68 caused 227% increase in the rate of dye fading at 21.1° and an increase of 271% at 48.8°. These observations may be associated with the fact that different lots of surfactant showed different peroxide values and variable pH behavior when aged in aqueous solution. Kuramoto, *et al.*, have reported similar observations for the effects of sorbitol on FD&C Blue No. 2. They attributed these effects to the variable concentration of catalyst present in different lots of sorbitol.

Further experiments with the blue and red system

In all cases reported in Table I, a significant drop in sample pH from the initial values between 6.5–7.0 was obtained after aging. The final pH fell in the range of pH 3.5 to 5.0, although there was considerable variation between each surfactant system. Similar observations were obtained for the surfactant solutions containing no dye. In the case of the ester-type surfactants (Tween 20 and Myrj 52) this pH drop is believed to be the result of hydrolysis. This may, in turn, explain the appearance of turbidity in several of the Tween 20 samples. For the ether surfactants, the pH drop on aging is more difficult to explain. No indications of microbiological contamination were found in any of the fourteen-day samples.

In order to eliminate the possibility that dye fading was associated with the increase in acidity in aged samples, all dye-surfactant combinations were reprepared in buffer solution. The results of this study are shown in Table II. It is apparent that, while the pH has remained constant over the storage interval, accelerated fading still occurs. We have deliberately avoided making a comparison of the fading effects in the buffered and unbuffered solutions because different lots of surfactants were used for each study.

The exact interactions between dye and surfactant which lead to accelerated fading are not known. It would appear from the results in Tables I and II that some characteristic common to all of the surfactants is involved in the fading mechanism. Since all of the surfactants studied are ethylene oxide addition products, it seemed worthwhile to examine the effects of other ethylene oxide condensates on dye stability. Limited experiments using polyethylene glycol 200, polyethylene glycol 600, and polyethylene glycol 6000, showed that these materials also cause accelerated fading of the certified dyes. The reaction of dyes with surfactants may result therefore because of the presence of the ethylene oxide moiety in the surfactants. While we do not postulate a complexing phenomenon at this time, Waibel (8) has reported that (ethoxylated) nonionic surfactants exist in solution as polyoxonium compounds which actually are not nonionic, but weakly cationic. He states that these positively charged ions are capable of reacting with anionic coloring agents.

Figure 1 shows that the rate of fading of FD&C Red No. 1 in the presence of Brij 35 proceeds by two pseudo first order reactions.<sup>7</sup> The first reaction, which is comparatively slow, was observed at all temperatures. This was followed at temperatures above 48.8° by a more rapid second reaction. Table III presents the rate constants at the five temperatures studied.

It is important to note further that FD&C Red No. 1 in the absence of Brij 35 did not fade at any of the temperatures investigated. The accelerating effects of the surfactant, therefore, are clearly evident.

The Arrhenius plots for the Red-Brij system are shown in Figs. 2 and 3. These plots suggest that two mechanisms are involved in the fading reaction. At temperatures below 37.5° we find the first reaction which is characterized by an energy of

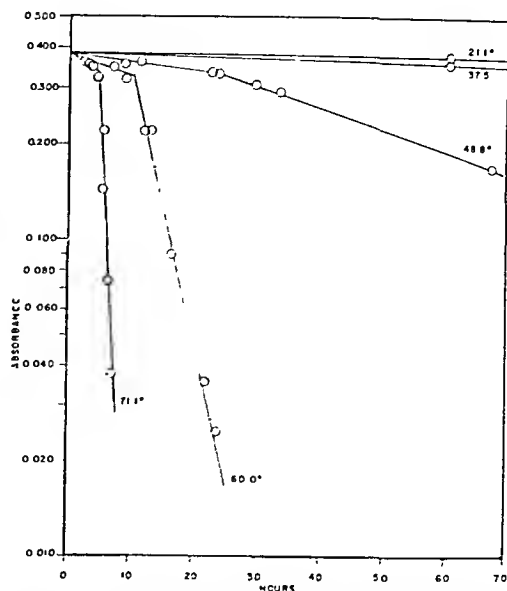


Fig. 1.—First order plots for color fading in FD&C Red No. 1-Brij 35 system.

TABLE III.—RATE CONSTANTS FOR THE FADING OF COLOR IN FD&C RED NO. 1-BRIJ 35 MIXTURES AT VARIOUS TEMPERATURES

Composi- tion <sup>a</sup>	Tempera- ture, °C., ±0.5° C.	Rate Constants (Reciprocal Hours)	
FD&C Red No. 1 (0.001%)	21.1	<1.0 × 10 <sup>-5</sup>	
	37.5	<1.0 × 10 <sup>-5</sup>	
	48.8	<1.0 × 10 <sup>-5</sup>	
	60.0	<1.0 × 10 <sup>-5</sup>	
	71.1	<1.0 × 10 <sup>-5</sup>	
FD&C Red No. 1 (0.001%) plus Brij 35 (1.0%)	21.0	1.64 × 10 <sup>-4</sup>	First Reaction Second Reaction
	37.5	1.25 × 10 <sup>-3</sup>	...
	48.8	5.94 × 10 <sup>-3</sup>	1.53 × 10 <sup>-3</sup>
	60.0	1.51 × 10 <sup>-2</sup>	1.85 × 10 <sup>-1</sup>
	71.1	3.89 × 10 <sup>-2</sup>	8.09 × 10 <sup>-1</sup>

<sup>a</sup> Diluent: Sorensen's M/15 phosphate buffer, pH 6.98.

activation of 21.9 Kcal./mole and a probability factor of  $3.8 \times 10^{-12}$ . At higher temperatures the second reaction occurs which has an energy of activation of 47.0 Kcal./mole and a probability factor of  $1 \times 10^{30}$ . These values are viewed with some reservation because replicate experiments showed that the kinetics may be altered significantly by what we believe to be lot to lot variations in the surfactant.

Figures 4 and 5 show that the rate of fading of FD&C Blue No. 2 in the absence and presence of Pluronic F-68 also follows pseudo first order kinetics. In this system, no induction period was obtained, although Kuramoto, *et al.*, have previously reported its presence (6). Table IV summarizes the rate data and Arrhenius plots for these systems are presented in Fig. 6.

It is apparent that in this particular study Pluronic F-68 produces a negligible effect on the color stability of the blue dye. In replicate studies,

<sup>7</sup> At the lower temperatures, observations were extended considerably beyond that shown in Fig. 1.

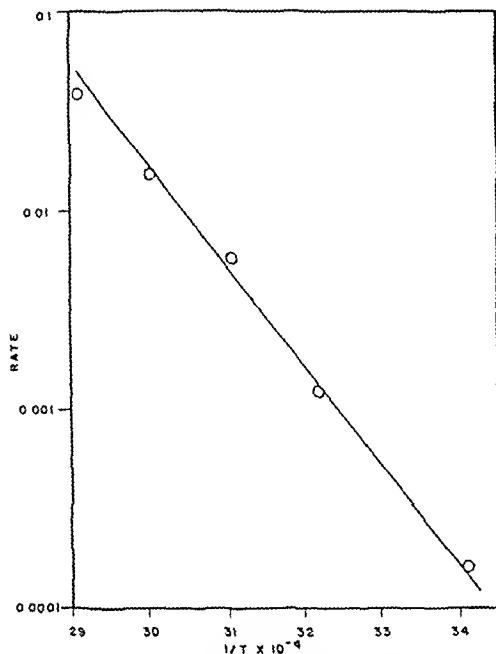


Fig. 2.—Arrhenius plot for first fading reaction in FD&C Red No. 1-Brij 35 system.

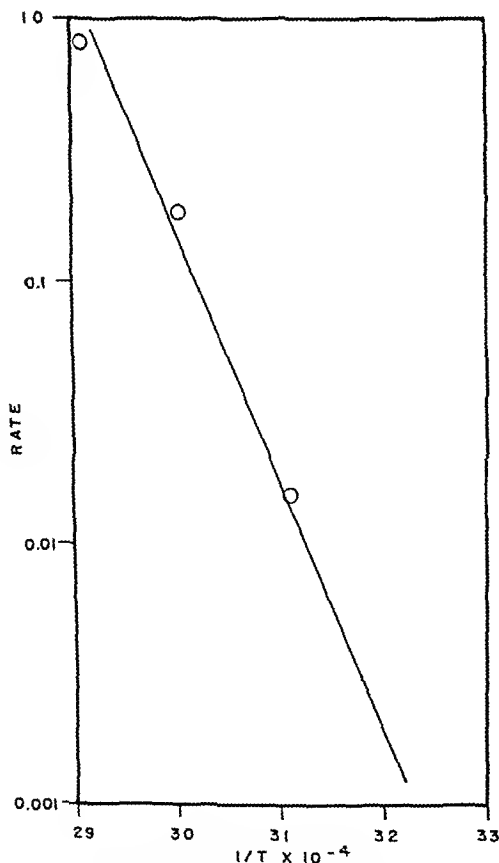


Fig. 3.—Arrhenius plot for second fading reaction in FD&C Red No. 1-Brij 35 system.

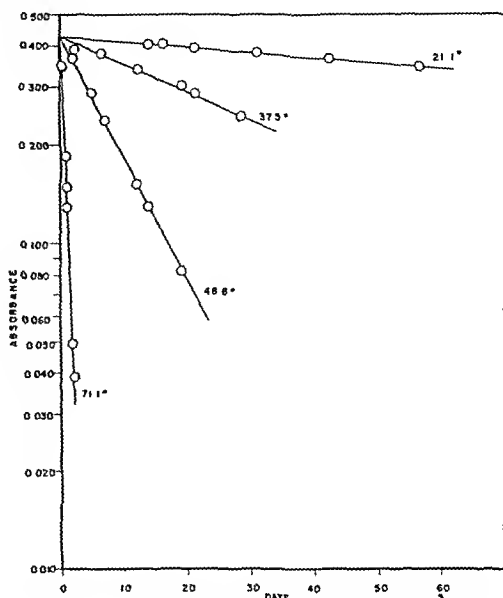


Fig. 4.—First order plots for color fading of FD&C Blue No. 2.

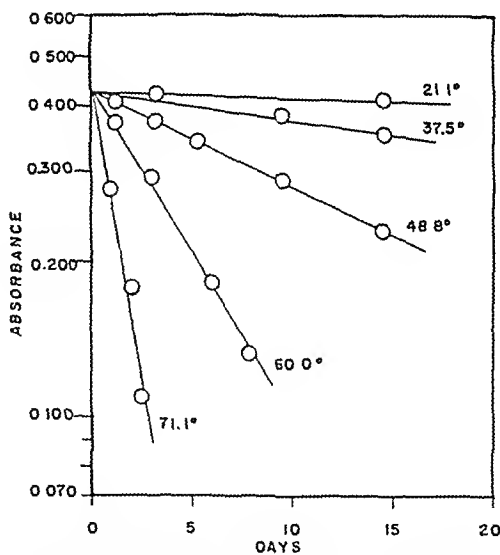


Fig. 5.—First order plots for color fading in FD&C Blue No. 2-Pluronic F-68 system.

we again noted that different kinetics may be obtained. For example, in one study the presence of Pluronic F-68 caused 227% increase in the rate of dye fading at 21.1° and an increase of 271% at 48.8°. These observations may be associated with the fact that different lots of surfactant showed different peroxide values and variable pH behavior when aged in aqueous solution. Kuramoto, *et al.*, have reported similar observations for the effects of sorbitol on FD&C Blue No. 2. They attributed these effects to the variable concentration of catalyst present in different lots of sorbitol.

Further experiments with the blue and red system

TABLE IV.—RATE CONSTANTS FOR THE FADING OF COLOR IN FD&amp;C BLUE NO. 2-PLURONIC F-68 MIXTURES AT VARIOUS TEMPERATURES

Composition <sup>a</sup>	Temperature ±0.5° C	Rate Constants (Reciprocal Hours)	Energy of Activation	Probability Factor
FD&C Blue No. 2 (0.001%)	21.1	$1.59 \times 10^{-4}$	22.4 Kcal /Mole	$6.3 \times 10^{12}$
	37.5	$7.99 \times 10^{-4}$		
	48.8	$3.52 \times 10^{-3}$		
	60.0			
	71.1	$4.18 \times 10^{-2}$		
FD&C Blue No. 2 (0.001%) plus Pluronic F-68 (1.0%)	21.1	$8.57 \times 10^{-4}$	22.7 Kcal /Mole	$6.3 \times 10^{12}$
	37.5	$4.77 \times 10^{-4}$		
	48.8	$1.95 \times 10^{-3}$		
	60.0	$6.61 \times 10^{-3}$		
	71.1	$2.09 \times 10^{-2}$		

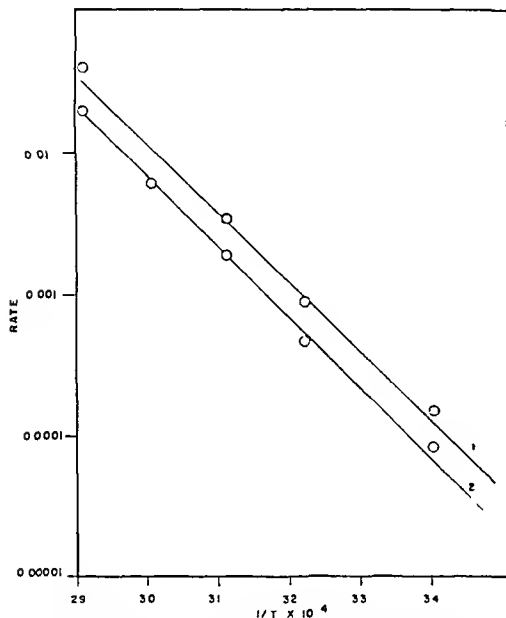
<sup>a</sup> Diluent: Sorensen's M/15 phosphate buffer, pH 6.98

Fig. 6—Arrhenius plots for fading reaction in: (1) FD&amp;C Blue No. 2-Pluronic F-68 system, (2) FD&amp;C Blue No. 2 solution

showed that the rate of color fading was not affected by preheating the surfactants before addition of the dye. These observations indicate that the mechanism of fading does not involve (stable) decomposition products of the surfactant.

### CONCLUSIONS

The results of this study show that nonionic surfactants may have a deleterious effect on the color stability of certified dyes. The intensity of this effect has been found to vary from one dye-surfactant system to another and is not generally

predictable. We believe further that variations in the lot to lot properties of surfactants may be an important factor in the color fading reactions.

While we have not attempted to postulate a mechanism for the fading reactions, our results suggest that interactions between the ethylene oxide moiety of the surfactant and the chromophoric groups of the certified dyes may be involved. This conclusion is confirmed in part by the fact that various polyethylene glycols also showed accelerating effects on the color fading of the certified dyes. We do not discount the possibility that trace contaminants which may appear in the surfactants and polyols as a result of the ethoxylation techniques are involved also.

In general, the degree of fading caused by nonionic surfactants in colored, multicomponent, pharmaceuticals and cosmetics will be less than that observed in the simple aqueous solutions studied in the present report. Our results do show, however, that interactions between surfactants and dyes should be carefully considered when color stability problems arise in products containing these materials.

### REFERENCES

- (1) Zuckerman, S., "Atlas Certified Dyes for Foods, Drugs and Cosmetics," H. Kohnstamm and Co., Inc., New York, N. Y., 1949.
- (2) Peacock, W. H., "The Coloring of Pharmaceutical Preparations," Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J., 1952.
- (3) Peacock, W. H., "The Application Properties of the Certified Coal Tar Colorants," Calco Chemical Division, American Cyanamid Co., Bound Brook, N. J., 1952.
- (4) Selar, R. N., and Freeman, K. A., *J. Assoc. Offic. Agr. Chemists*, **37**, 905 (1954).
- (5) Selar, R. N., and Freeman, K. A., *ibid.*, **37**, 913 (1954).
- (6) Kuramoto, R., Lachman, L., and Cooper, J., *This Journal*, **48**, 175 (1958).
- (7) Karrer, P., "Organic Chemistry," Elsevier Publishing Co., New York, N. Y., 1950, p. 572.
- (8) Waibel, H., *Mitt. chem. Forschungsinst. Wirtsch. Osterr.*, **9**, 34 (1955).

# The Relationship Between Physiological Availability of Salicylates and Riboflavin and *In Vitro* Disintegration Time of Enteric Coated Tablets\*

By A. B. MORRISON and J. A. CAMPBELL

Studies were conducted to relate the *in vitro* disintegration time of enteric coated tablets with physiological availability of salicylates and riboflavin, as determined by the urinary excretion of these drugs by human subjects. *In vitro* disintegration times were determined by the procedure given in U. S. P. XV Second Supplement, modified by the use of sixty minutes time in simulated gastric juice. Rates of urinary excretion of the drugs were determined after dosing subjects with seven salicylate preparations and five riboflavin preparations. Two products were found which disintegrated after less than thirty minutes in simulated gastric juice, while a third product was extremely resistant to the simulated digestive juices, and, in fecal recovery studies, was recovered intact from the feces. Studies on rate of urinary excretion of the drugs indicated delayed absorption from the enteric coated preparations. Salicylate tablets with *in vitro* disintegration times as long as two hundred and thirteen minutes were fully available *in vivo*, whereas a riboflavin preparation with an *in vitro* disintegration time of one hundred and twenty-eight minutes was only 41% available *in vivo*. It is suggested that enteric coated tablets should withstand the action of simulated gastric juice for at least sixty minutes, to ensure that these preparations do not disintegrate in the stomach. Furthermore, until quantitative *in vivo* data are available for individual drugs, it is suggested that to ensure full availability enteric coated preparations, other than salicylates, should disintegrate within thirty minutes in simulated intestinal juice.

ALTHOUGH ENTERIC COATINGS have been used on tablets for many years, surprisingly little work has been done to evaluate their effectiveness. Most reports on enteric coated preparations have described the composition of various coating mixtures, or have dealt with the results of *in vitro* disintegration tests. Little attention has been paid to *in vivo* availability in man. Kanig (1) reported that the *in vitro* disintegration times of enteric coated tablets taken from the same container varied from fifteen minutes to four hours. He concluded that "before enteric coatings may take their place among the reputable dosage forms, it is highly desirable that standardized testing procedures be instituted, in order to place the level of investigations in this field on a rational, easily duplicated, and thoroughly reliable basis." Crisafio, *et al.* (2), found that 31 of 64 samples of enteric coated preparations failed completely to resist the action of simulated gastric juice. Most of the products tested disintegrated within an hour in simulated intestinal juice. Trivedi (3) studied the disintegration of enteric coated tablets by use of an *in vitro* test similar to that given in U. S. P. XV (4) and observed wide variation in the disintegration time of tablets from sample to sample, and, in many instances, from tablet to tablet in the same

sample. Tablet recovery from stools indicated a frequent loss of medication. Trivedi concluded that present U. S. P. standards are inadequate to rule out many faulty enteric coatings, and went so far as to suggest that enteric coatings might well be abandoned in favor of more reliable dosage forms.

Blythe, *et al.* (5), pointed out recently that *in vitro* tests alone cannot be used to predict the clinical effectiveness of enteric coated products. They stressed the need for studies on the relationship between *in vitro* data and quantitative *in vivo* results. Blood acetylsalicylic acid levels found with an enteric coated preparation were similar to those obtained with equivalent amounts of plain acetylsalicylic acid, indicating full availability of the medication *in vivo*.

Previous studies from this laboratory (6-9) showed that sugar coated tablets which did not disintegrate within sixty minutes (thirty minutes in simulated gastric fluid and the remainder of the time in simulated intestinal fluid) did not allow all of their contained drug to be released in human subjects, as measured by urinary excretion of riboflavin and *p*-aminosalicylate. The present experiments were conducted to study the relationship between *in vitro* disintegration time of enteric coated tablets and physiological availability of salicylates and riboflavin. Data were also obtained on variation in excretion rate over a period of time after dosing with the various preparations tested.

\* Received October 2, 1959, from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa, Ontario, Canada.

The authors are indebted to the subjects who took part in this investigation, and to Mr. C. Perusse for technical assistance.

## METHODS

**In Vitro Disintegration Times**—The apparatus and fluids described in U S P XI (4) and the fluted disks described in U S P XV Second Supplement (10) were used to determine disintegration time. The tablets were immersed in simulated gastric fluid for sixty minutes, and the remainder of the time in simulated intestinal fluid. Disintegration times reported were mean times of at least two separate tests on six tablets each, for tablets tested *in vivo* and *in vitro*, and of at least one test on six tablets for preparations tested only *in vitro*.

**Physiological Availability**—Riboflavin or apparent salicylate levels were determined in the urine prior to dosing and after administration of standard or test preparations. Total salicylate was determined by a modification of the ferric nitrate method of Trinder (11), and riboflavin was determined by the U S P fluorometric procedure (4). Studies were made on the stability of the salicylate in urine held under various conditions. The specificity of the method used for salicylate determination was checked by comparing the results found with those obtained by the method of Smith, *et al* (12), which determines free salicylate, total salicylate, and salicylic acid, separately. The amounts of salicylate or riboflavin obtained in the urine after dosing were corrected by subtracting the appropriate blank value determined on the urine of the same subjects. As in previous studies (6-9), physiological availability was calculated in the following manner:

Percentage physiological availability =

$$\frac{\text{Per cent excretion from preparation}}{\text{Percentage excretion from standard}} \times 100$$

**Subjects**—Four to nine normal male subjects, ranging in age from 26 to 45 years, were used in the experiments. While on test, they continued to consume their regular diets, but were cautioned to eat meals similar in nature from day to day. During the riboflavin studies, the subjects refrained from eating foods high in riboflavin, such as liver.

**Salicylate and Riboflavin Standards**—The subjects received doses of 10 gr (648 mg) or 15 gr (972 mg) of acetylsalicylic acid, or 5 mg of riboflavin, in rapidly disintegrating standard tablets.

**Commercial Preparations Tested**—Seven commercial enteric coated salicylate preparations, and five commercial enteric coated products containing riboflavin, were tested *in vivo*. In addition, six other salicylate preparations, and two other riboflavin preparations, were tested *in vitro*, but not *in vivo*. Salicylate preparations A, B, D, and J were samples from different lots of one product. Riboflavin preparations O and T were samples from different lots of the same product, as were preparations P and R. Each subject received from 10 to 20 gr (648 to 1,296 mg) of salicylate, or 3 mg of riboflavin, from the various preparations. All products were assayed for salicylate or riboflavin prior to administration.

**Assay Periods**—The standards or commercial preparations were given at 8:45 a.m. after breakfast. Urine was collected in opaque bottles after two, four, six, eight, fourteen, and twenty-four

hours, and at four hour intervals thereafter until the rate of excretion approached blank values. For the riboflavin studies, 2 ml of 3.5 N H<sub>2</sub>SO<sub>4</sub> was added to each bottle, to ensure stability of the vitamin.

**Fecal Recovery Studies**—Three subjects were each given 10 tablets (6.5 Gm) of salicylate preparation C. The feces were collected for up to seventy hours after ingestion of the tablets. Undissolved tablets were separated from the feces by gently brushing the stool sample through a screen.

## RESULTS

**Salicylate Studies**—Studies on the validity of the method used for salicylate determination showed that the values found for total salicylate by a modification of the method of Trinder (11), were in good agreement with those obtained by the procedure of Smith, *et al* (12). The latter method utilizes an ethylene dichloride extraction and determines free salicylate, total salicylate, and salicylic acid, separately. Since the modified Trinder method is easily carried out on large numbers of urine samples, it was used in the present experiments. The stability of salicylate in urine was studied by collecting urine from a subject dosed with 975 mg acetylsalicylic acid and storing the urine overnight at room temperature, or in the refrigerator. Aliquots of the sample held at room temperature were treated by layering with toluene or were acidified with 3% of 3.5 N H<sub>2</sub>SO<sub>4</sub>. The values found for acetylsalicylic acid (determined as salicylate) in mg per ml of urine were as follows: fresh urine, 1.11; refrigerated urine, 1.04; urine held at room temperature, 1.08; acidified urine held at room temperature, 1.02; toluene treated urine, 1.03. Since the results showed salicylate in urine to be stable at room temperature overnight, it was not felt necessary to refrigerate the samples or to acidify them.

Descriptive data on the salicylate preparations used are summarized in Table I. Of the products tested *in vivo*, two contained sodium salicylate, while the remainder contained acetylsalicylic acid. The *in vitro* disintegration times of the salicylate products varied from twenty-seven minutes (product G) to over four hundred and eighty minutes (product C). As is indicated from the disintegration time, product G was unable to withstand the action of gastric fluid. Product C, on the other hand, was extremely resistant to digestion. Some preparations (e.g., products G and K) showed wide variation in *in vitro* disintegration time of tablets taken from the same container, whereas other products (e.g., products A, B, N) showed little variation from tablet to tablet.

The data on urinary excretion of acetylsalicylic acid (measured as salicylate) after ingestion of 650 mg or 975 mg of the standard dose are summarized in Table II. The mean excretion in four trials varied from 74.6 to 89.2%, with an overall mean of 84.4%. The latter value agrees well with results on salicylate excretion reported in the literature (13). The mean percentage excretion in trial I (975 mg dose) was less than that in the other three trials (650 mg dose). The decrease is not believed to be related to dosage level, however, since the results were not consistent

TABLE I.—DESCRIPTION OF ENTERIC COATED SALICYLATES STUDIED

Product	Medication	<i>In Vitro</i> Disintegration Time, min.	Salicylate per Unit, gr.	Salicylate per dose, gr.
A	Acetylsalicylic acid	87 ± 5.0 <sup>a</sup>	5.0	15.0
B	Acetylsalicylic acid	86 ± 4.4	5.0	15.0
C	Acetylsalicylic acid	480 ± ...	10.0	20.0
D	Acetylsalicylic acid	110 ± 27.1	5.0	15.0
E	Sodium salicylate	213 ± 21.3	10.0	10.0
F	Sodium salicylate, <i>p</i> -aminobenzoic acid, ascorbic acid	145 ± 8.9	5.0	15.0
G	Acetylsalicylic acid, ascorbic acid	27 ± 23.1	10.0	10.0
H	Acetylsalicylic acid	75 ± 4.0	5.0	...
J	Acetylsalicylic acid	111 ± 22.7	5.0	...
K	Acetylsalicylic acid	76 ± 30.3	5.0	...
L	Salicylic acid, <i>p</i> -aminobenzoic acid	100 ± 16.1	3.75	...
M	Sodium salicylate, <i>p</i> -aminobenzoic acid, ascorbic acid	100 ± 10.8	5.0	...
N	Salicylic acid, <i>p</i> -aminobenzoic acid	117 ± 5.9	3.75	...

<sup>a</sup> Standard deviation.

TABLE II.—PERCENTAGE OF STANDARD DOSE OF ACETYSALICYLIC ACID RECOVERED IN URINE

Subject	Trial 1 15-gr. dose	Trial 2 10-gr. dose	Trial 3 10-gr. dose	Trial 4 10-gr. dose
ABM	84.1	78.4	81.6	...
JAC	59.2	...	...	78.1
CP	87.8	95.4	...	90.0
MM	67.5	89.6	93.4	92.5
CR	70.7	93.6	92.2	88.5
TKM	96.2	100.2	99.1	96.2
KS	59.3	87.5	70.7	...
TP	78.1	86.9	...	...
DS	68.3	60.4	98.5	77.4
Mean	74.6	86.5	89.2	87.1

TABLE III.—PHYSIOLOGICAL AVAILABILITY OF SALICYLATE IN SEVEN PRODUCTS<sup>a</sup>

Subject	Per Cent A	Per Cent B	Salicylate C	Available in D	Product E	Product F	Product G
ABM	51	89	0	111	84	...	110
JAC	107	106	...	181	124	133	...
CP	85	80	26	94	...	94	119
MM	102	112	4	...	112	...	107
CR	76	92	0	91	112	99	136
TKM	88	73	2	105	123	101	130
KS	95	108	90	57	119	104	123
DS	93	100	41	114	136	112	84
TP	...	...	...	109	...	106	...
Mean	87	95	23	101	116	107	116
<i>In Vitro</i> disintegration time, min.							
	87	86	480+	110	213	145	27

<sup>a</sup> Products A, B, and D were samples from different lots of the same product.

from subject to subject; some subjects (e. g., ABM, CP) showed relatively constant percentage excretion from the standard dose in repeated trials, whereas others were more variable. Because of the intersubject and intertrial variation in percentage excretion, the average of all trials was used in calculating physiological availability on each subject.

The average blank excretion of apparent salicylate in the urine without dosing varied from 0.10 mg. to 0.16 mg. per minute at the various collection periods.

The results of the physiological availability studies with the salicylate preparations are summarized in Table III. Excretion values for products containing sodium salicylate were expressed in terms of acetylsalicylic acid. Considerable variation was noted between subjects. Despite the wide range in *in vitro* disintegration times (Table I), all of the preparations except preparation C, were available physiologically. The latter preparation, which had an *in vitro* disintegration time exceeding four hundred and eighty minutes, was only 23% available on the average. This value is somewhat misleading, since the product was essentially unavailable to four of the seven subjects who consumed it. Subject KS, however, showed a good availability of salicylate in this product (90%), illustrating the great variation encountered between subjects. Most of the subjects showed apparent availabilities of somewhat over 100% for the salicylate in products E, F, and G. Such variability is well known in biological assays.

Data on mean rate of excretion of salicylate after ingestion of the seven products were compared with

those for the combined standards in Fig. 1. As suggested by Swintosky, *et al.* (14), a semilog plot was used. None of the preparations showed a peak excretion rate as high as the standard, although all except product C were fully available physiologically. The peak of salicylate excretion occurred earlier with the standard than with any of the preparations studied. With the exception of product C, the excretion of salicylate was more sustained with the products tested than with the standard. Products A and B, which were two samples of the same preparation, showed peak net excretion rates of 0.57 and 0.48 mg./min. respectively.

Product C, which showed low availability, also showed low and nonsustained urinary excretion. The curve shown is actually somewhat misleading, since it represents average values, and the salicylate in the preparation was essentially unavailable to four of seven subjects. Prior to eight hours after ingestion of product C, there was no detectable net urinary excretion of salicylate. The rate of excretion found with product D declined at approximately the same rate as the standard, but the peak of excretion occurred later. Product E, which disintegrated slowly *in vitro* (two hundred and thirteen minutes), showed a peak excretion rate later than did the other products studied. The rate of excretion found with this product declined much



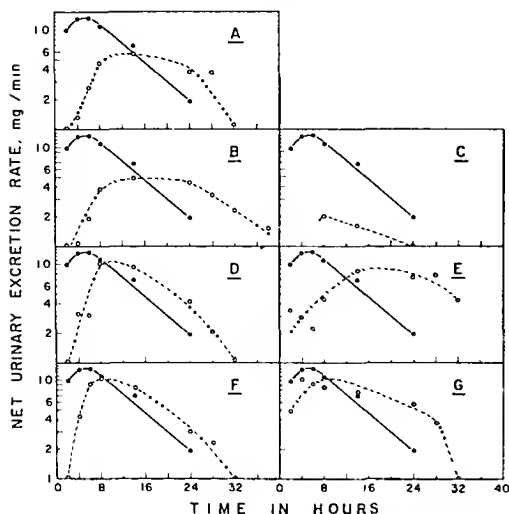


Fig. 1.—Urinary excretion curves of salicylate in seven tablets O—O—O—, compared with curve for a salicylate standard tablet ●—●—●.

more slowly than did that with the standard. Products F and G both showed peak excretion rates of approximately 1 mg./min. Although product G disintegrated *in vitro* after only twenty-six minutes in gastric juice, its peak excretion rate did not occur earlier than those of the other preparations.

The fecal recovery studies with salicylate product C showed that all three individuals who consumed 10 tablets of this preparation recovered the tablets in the feces. These results agree with those obtained by urinary excretion measurements, which also showed poor availability of this product. Tablets were recovered in as little as twelve hours after ingestion in one individual, and as late as seventy hours after ingestion in a second subject. A few of the recovered tablets showed cracks in the enteric coat, but all were intact, with only the sugar coat removed. In a few of the tablets which had passed through the gastrointestinal tract, the enteric coat was removed by hand. The core of the tablets was still extremely hard, and even in the absence of the enteric coat, would be expected to resist disintegration for some time.

**Riboflavin Studies.**—Descriptive data on the riboflavin-containing preparations studied are summarized in Table IV. *In vitro* disintegration times varied from twenty-four minutes (product V) to two hundred and forty-one minutes (product P). As with the salicylate products some of the riboflavin preparations (e. g. products P, T) showed wide variation in *in vitro* disintegration time of tablets from the same container, whereas other products were less variable. In all cases, the dose of riboflavin administered was 3.0 mg. Although it perhaps might have been desirable to increase the dose to the level of the standard, 5 mg., it was not convenient to do so, since most of the preparations contained high levels of iron. Product V, which disintegrated in gastric juice, could not be termed enteric coated.

Data on the percentage excretion of the standard dose of riboflavin were presented previously (9), and

those for blank excretion values were published elsewhere (15). Briefly, the mean excretion, in 5 trials, varied from 57 to 60%, with an overall mean of 58%.

The results of the physiological availability studies with the riboflavin preparations are summarized in Table V. In contrast to the results obtained with the salicylate preparations (Table III), only two of the riboflavin products had availability approaching 100%. Product O, which had an *in vitro* disintegration time of one hundred and four minutes, was 81% available *in vivo*. Preparation S, which disintegrated in eighty minutes *in vitro*, showed an average *in vivo* availability of only 75% in two trials. Products P, R, and T, which had disintegration times of two hundred and forty-one, one hundred and twenty-eight, and two hundred and forty-seven minutes were only 29, 41, and 40% available, respectively. The high variation noted between subjects with all products is typical of preparations on the borderline of *in vivo* availability (6, 9).

Data on rate of excretion of riboflavin after ingestion of the five products were compared with those for the standard in Fig. 2, using a semilog plot. Since only 3 mg. of each of the riboflavin preparations were ingested, the rate values calculated were multiplied by 1.67 to make them comparable to the 5 mg. standard. The peak urinary excretion rate was directly related to physiological availability, and occurred earlier with the standard than with the products tested. Products O and S, which showed the highest physiological availability, also showed the highest peak excretion rates. Products P, R, and T, which were poorly available *in vivo*, showed peak excretion rates only 17 to 29% that of the standard. With the possible exception of product P, the rate of riboflavin excretion declined somewhat more slowly with the preparations tested than with the standard.

## DISCUSSION

The results of the present studies showed that tablets labeled as enteric coated exhibited widely differing *in vitro* disintegration times and physiological availabilities. Two preparations were found which disintegrated *in vitro* in simulated gastric juice. These products do not conform to any acceptable definition of enteric coated products, despite label claims. On the other hand, tablets of salicylate product C were extremely resistant to disintegration and were recovered intact from the feces. It is obvious that this product would not exert the desired therapeutic effects. These findings would indicate that a significant proportion of commercially available enteric coated salicylates and multi-vitamin-mineral preparations do not satisfactorily fulfill the purpose for which they were intended.

It is interesting to note the difference in the *in vivo-in vitro* relationship found with salicylate and that found with riboflavin. Salicylate preparations with *in vitro* disintegration times as long as two hundred and thirteen minutes were fully available *in vivo*, whereas a riboflavin preparation with an *in vitro* disintegration time of one hundred and twenty-eight minutes was only 41% available *in vivo*. This difference may be due, in part, to the fact that salicylates are readily absorbed well down in the intestinal tract (13), whereas there is evidence that

TABLE IV—DESCRIPTION OF ENTERIC COATED VITAMIN PREPARATIONS STUDIED

Product	Medication	<i>In Vitro</i> Disintegration Time, min	Riboflavin per Unit, mg	Riboflavin per Dose, mg
O	Multivitamins and minerals	104 ± 9 7 <sup>a</sup>	1 0	3 0
P	Homatropine, caffeine, vitamins, and ammonium chloride	241 ± 58 6	1 0	3 0
R	Homatropine, caffeine, vitamins, and ammonium chloride	128 ± 7 2	1 0	3 0
S	Multivitamins and minerals	80 ± 6 4	1 0	3 0
T	Multivitamins and minerals	247 ± 35 6	1 0	3 0
U	Multivitamins and minerals	114 ± 8 5	2 5	
V	Multivitamins and minerals	24 ± 3 3	1 0	

<sup>a</sup> Standard deviationTABLE V.—PHYSIOLOGICAL AVAILABILITY OF RIBOFLAVIN IN FIVE PRODUCTS<sup>a</sup>

Subject	Per Cent Riboflavin Available in Product				
	O	P	R	S <sup>b</sup>	T
ABM	97	0	23	33	20
JAC	113	44	29	80	24
CP		30			
MM	85		69		
TKM	73		12	109	72
KS	36	37	66	78	45
DS	..	33	45		
Mean	81	29	41	75	40
<i>In Vitro</i> disintegration time, min.	104	241	128	80	247

<sup>a</sup> Products O and T were samples from different lots of the same product, as were products P and R<sup>b</sup> Mean of two trials.

riboflavin is destroyed in the lower portion of the intestine (16).

The variation between subjects in percentage physiological availability of the preparations tested was probably a reflection, in part, of the well-known variation in clinical response of patients to doses of drugs. It was probably influenced also by the variation in disintegration time of tablets taken from the same container. For some products, the variation in *in vitro* disintegration time between tablets was very large, indicating the need for improved production control procedures. It is of interest to note that the subjects used by Blythe, *et al* (5), showed considerable variation in blood salicylate levels, probably, in part, due to the factors mentioned above.

On the question of time limits for *in vitro* disintegration of enteric coated tablets, it is obvious that all products should withstand the action of simulated gastric juice for at least sixty minutes. In fact, Blythe, *et al* (5), recommended that enteric coated tablets should resist attack by simulated gastric fluid for at least four hours.

Although it is difficult, from the data available, to propose exact time limits for the disintegration of enteric coated salicylate preparations in simulated intestinal juice, there seems to be no reason why such products should not disintegrate within about four hours. On the other hand, it appears that enteric coated riboflavin preparations must disintegrate within about thirty minutes in simulated intestinal juice, to show full physiological availability. Thus, when tested *in vitro* by the procedure used in the

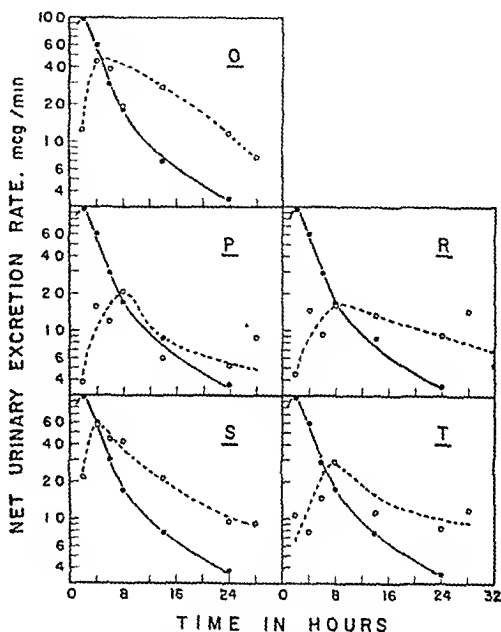


Fig 2—Urinary excretion curves for riboflavin in five tablets O—O—O—O, compared with curve for a riboflavin standard tablet ●—●.

present studies, enteric coated vitamin preparations containing riboflavin should disintegrate within ninety minutes (sixty minutes in simulated gastric juice and thirty minutes in simulated intestinal juice). This time limit would correlate well with earlier work on riboflavin (6, 8, 9), in which it was found that, to be fully available, sugar coated tablets must disintegrate within sixty minutes (thirty minutes in simulated gastric juice and the remainder of the time in simulated intestinal juice).

Further information is required on the relationship between *in vivo* availability and *in vitro* disintegration time for other enteric coated preparations. Until such information is available, however, it would seem advisable to suggest that enteric coated preparations (other than salicylates) should disintegrate in simulated intestinal juice within the time limit suggested for riboflavin, i. e. thirty minutes. This proposal may need to be modified as quantitative *in vivo* information becomes available for specific drugs.

## REFERENCES

- (1) Kanig, J. L., *Drug Standards*, 22, 113(1954).
- (2) Crisafio, R., Taylor J., and Chatten, L. G., *ibid.*, 23, 1(1955).
- (3) Trivedi, A. H., M.S. Thesis, University of Southern California, 1958.
- (4) "U. S. Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955.
- (5) Blythe, R. H., Grass, G. M., and MacDonnell, D. R., *Am. J. Pharm.*, 131, 206(1959).
- (6) Chapman, D. G., Crisafio, R., and Campbell, J. A., *THIS JOURNAL*, 43, 297(1954).
- (7) Chapman, D. G., Crisafio, R., and Campbell, J. A., *ibid.*, 4S, 374(1956).
- (8) Chapman, D. G., Chatten, L. G., and Campbell, J. A., *Can. Med. Assoc. J.*, 76, 102(1957).
- (9) Morrison, A. B., Chapman, D. G., and Campbell, J. A., *THIS JOURNAL*, 48, 634(1959).
- (10) "U. S. Pharmacopeia," Second Suppl. to 15th rev., Mack Publishing Co., Easton, Pa., 1958.
- (11) Trinder, P., *Biochem. J.*, 57, 301(1954).
- (12) Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalek, S., *J. Pharmacol. Exptl. Therap.*, 87, 237(1946).
- (13) Gross, M., and Greenberg, L. A., "The Salicylates," Hillhouse Press, New Haven, Conn., 1948.
- (14) Swintosky, J. V., Robinson, M. J., and Foltz, E. L., *THIS JOURNAL*, 46, 403(1957).
- (15) Morrison, A. B., Cox, C. E., and Campbell, J. A., *Can. Dietet. Assoc. J.*, 22, 58(1960).
- (16) Selye, H., *J. Nutrition*, 25, 137(1943).

## Emulsification With Ultrasonic Waves I\*

### Evaluation of the Minisonic and a Modified Analysis Procedure

By ROBERT E. SINGISER† and HAROLD M. BEAL

A modified photomicrography procedure has been developed to speed the size-frequency method of analyzing emulsions. This method employs a film strip and rule simultaneously projected. Classification of 1,000 oil globules into the proper size intervals can be done in about one-half of the time formerly required. The Minisonic homogenizer was investigated for (a) the effect of long insonation periods on emulsions and (b) various methods of manufacturing the emulsion. No coagulation of emulsions occurred when exposed to ultrasonic waves for thirty minutes.

RECENTLY ultrasonic equipment operating on the "liquid whistle" principle has been made commercially available. This type of apparatus, for the first time, makes practical ultrasonic emulsification in production quantities. The laboratory size model of this apparatus, the Minisonic,<sup>1</sup> was evaluated in this investigation. The effect of prolonged exposure of emulsions to high frequency sound and the optimum method of producing the emulsion were studied.

Unless simple gross observations are to be relied upon, a size-frequency analysis is necessary to evaluate the emulsion. From the analysis data, numerous parameters may be calculated. A simpler, faster method for a size-frequency analysis of emulsions has been perfected.

#### ANALYSIS TECHNIQUES

In a size-frequency analysis at least 1,000 internal phase globules are usually measured. This has varied from 50 to 50,000, with most investigators using the 1,000-4,000 range. Statistically, the reliability of the results varies with the square root of the number of counts made. Harkins and Bee-man (1) carried out the first size-frequency analysis on emulsions in 1929. A microscopic mounting of the emulsion was projected onto a screen; then the individual globule images were traced and measured. Others have determined globule diameters from photomicrographs (2) or by projecting the microscopic field onto drawing paper with a camera lucida attachment; then tracing the globule outlines (3, 4). These procedures were reported to require a minimum of one to one and one-half hours to measure 1,000 globules. Often an assistant is required. With the exception of the photomicrographic procedure no permanent record was obtained.

The method of analysis used in this study was quicker and easier than those previously reported. A permanent record was also obtained.

**Equipment.**—A Helber counting chamber and standard hemocytometer cells were used to mount emulsions for microscopic examination. In all of the cells the central portion is ruled into squares

\* Received August 21, 1959, from Abbott Laboratories, Scientific Division, North Chicago, Ill.

This paper is based on a dissertation submitted by Robert E. Singiser to the Graduate School of the University of Connecticut in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Fellow, American Foundation for Pharmaceutical Education.

<sup>1</sup> Sonic Engineering Corp., 146 Selleck St., Stamford, Conn.

0.0025 mm.<sup>2</sup> in area (50 × 50 μ). A Bausch and Lomb microscope having a self-contained lighting source was employed, utilizing the 43× high power lens. The light intensity could be controlled with a diaphragm and/or a transformer.

A Leitz Micro-Ibso attachment with a 35-mm. Leica camera was placed on the microscope. This setup permits quick and accurate photomicrography. It is possible to prepare, mount, and photograph a large number of emulsions per day and to analyze the emulsions at a more convenient time.

The emulsions were diluted sufficiently for easy counting, generally from 1:100 to 1:450. The diluted emulsion was allowed to stand for thirty to forty-five minutes so that the dispersed globules would rise to a single plane. Photographs of three to four fields on the ruled cell were taken.

After the film was developed, it was projected directly on a screen via a strip film projector. At the same time, a horizontal rule was projected. The rule was composed of vertical lines, the distance between each representing 5 μ when projected. The rule was permanently mounted in the projector. This arrangement allowed the rule and film frame to be in focus simultaneously. The usual magnification was approximately 5,000 diameters. The horizontal rule was standardized against a photograph of the Helber cell where the width of the squares is known to be 50 μ.

**Measuring Technique.**—In the analysis of the globules, the exact diameters were not recorded. Class limits of 1 μ were selected, and the oil globule diameters were recorded as frequency of occurrence within the class limits. It was found that despite the final magnification of 5,000× the optics of the microscope-camera device precluded differentiating between globules less than 1 μ and those of 1–2 μ. Therefore, the first two class intervals had to be combined into one 0–2 μ class interval. Any globules greater than 10 μ in diameter were included in the 10–11 μ class and arbitrarily assigned an average diameter of 10.5 μ.

The film strip was passed down over the rule, and each globule was measured as it fell on the rule. Measurements were made on only a small vertical strip of the film at each pass over the rule. The measurements were recorded on an eight key blood cell calculator. Each key represented a class interval (e. g., 0–2, 2–3, 3–4 μ, etc.). As a globule was measured, it was recorded by depressing the properly designated key. Each key totaled individually. It was not necessary to look away from the screen to record the measurement; appreciably speeding up the analysis. As ten class intervals were used it was necessary to record by hand two of the less frequently used intervals. Measurements of 1,000 or more globules could easily be made in twenty-five to forty-five minutes, depending on the dispersion within the sample. This effected a considerable saving in time.

**Reproducibility.**—The reproducibility of the method of analysis was checked by making three complete measurements on two different emulsions. One emulsion was very uniform in dispersion, the second emulsion quite coarse with globules distributed throughout the counting range. The results of the triplicate measurements are given in Table I. The mean globule diameter ( $d_{ar}$ ), stand-

ard deviation ( $s$ ), and specific interfacial area ( $S$ ) were calculated.

### Minisonic Homogenizer

The Minisonic apparatus is a hydrodynamic device which operates on the Pohlmann "liquid whistle" principle. It has the advantages over other ultrasonic generators of: (a) having a continuous flow, (b) operating directly in the liquid with little energy loss, and (c) using the relatively low frequency of 22 kc. Alexander (5) believes oxidation and depolymerization reactions are held to a minimum at low frequencies.

The Minisonic has a single or double funnel arrangement which holds the emulsion components and acts as a reservoir for the gear pump. Heat of 60–70° is generated in the liquid if it is recycled for long periods. To avoid this cavitation heating, a water-cooled jacket was constructed around the outer funnel. This arrangement permitted recycling for thirty minutes while maintaining the temperature at 35°.

### Methods of Manufacture Studied

The manufacturer of the Minisonic suggests that emulsions be prepared using a two-funnel method where the continuous phase is placed in the outer funnel and recycled while the disperse phase is fed into it from the inner funnel. Oil-soluble and water-soluble additives are to be added in their respective phases.

The manufacturer's suggested method for making emulsions was compared with the method of Atlas Powder Co. (6) where both hydrophilic and lipophilic agents are added to the oil phase. Otherwise the two-funnel procedure was used.

A third method of preparation was also evaluated. This consisted of preparing coarse stock emulsions or premixes by hand shaking in a gallon bottle. Aliquots were then withdrawn and run in the Minisonic with the inner funnel removed.

**Emulsions Evaluated.**—Oil-in-water emulsions of 25% mineral, peanut, and safflower oils, each using a Span 80<sup>2</sup>/Tween 40<sup>2</sup> emulsifier system, were prepared by each of the three methods of manufacture. The total Span-Tween concentration was 4%, the relative amounts of each used was calculated from their respective HLB values of 4.3 and 15.6 for o/w emulsions of mineral oil (HLB 12), peanut oil (HLB 8), and safflower oil (HLB 11.5).

Similar emulsions of each oil were made with a Span 80/Pluronic F-68<sup>3</sup> emulsifier system of 4% total concentration. The relative amounts used were based on respective HLB values of 4.3 and 16.<sup>4</sup> The Atlas method of manufacture could not be evaluated in this instance since the Pluronic F-68 was not dispersible in the oil phase.

**Evaluation.**—The emulsions were insonated in the Minisonic homogenizer for fifteen minutes. Following this a size-frequency analysis was done as described. From these data the mean globule diameter ( $d_{ar}$ ), its standard deviation ( $s$ ), and the specific interfacial area ( $S$ ) were calculated. The results are shown in Table II.

<sup>2</sup> Product of Atlas Powder Co., Wilmington, Del.

<sup>3</sup> Product of Wyandotte Chemical Co., Midland, Mich.

<sup>4</sup> HLB values experimentally determined for safflower oil and Pluronic F-68, other values from the literature.

TABLE I—TRIPPLICATE MEASUREMENTS

Trial	—Peanut Oil/Acetic Emulsion—			—Safflower Oil/Pluronic Sp in Emulsion—		
	1	2	3	1	2	3
$N^a$	1,168	1,203	1,305	1,081	1,098	1,213
$d_{av}^b$	1.71 $\mu$	1.63	1.85	3.09	3.15	3.20
$s^c$	0.86 $\mu$	0.82	0.86	2.43	2.43	2.48
$S^d$	$2.39 \times 10^4$	$2.49 \times 10^4$	$2.35 \times 10^4$	$0.90 \times 10^4$	$0.91 \times 10^4$	$0.90 \times 10^4$

<sup>a</sup>  $N$  Total number of counts    <sup>b</sup>  $d_{av}$  arithmetic mean globule diameter    <sup>c</sup>  $s$  standard deviation     $s = \sqrt{\frac{n(\sum f^2) - (\sum f)^2}{n(n-1)}}$   
<sup>d</sup>  $S$  specific interfacial area cm<sup>2</sup>/ml

TABLE II—EFFECT OF THE VARIOUS METHODS OF INCORPORATING EMULSION COMPONENTS IN THE MINISONIC

Diameter $\mu$	Premix Single Funnel		O S/Inner Funnel <sup>a</sup> W T/Outer Funnel <sup>b</sup>		O S T/Inner Funnel <sup>c</sup> Water/Outer Funnel		Premix Single Funnel		O S/Inner Funnel <sup>a</sup> W T/Outer Funnel <sup>b</sup>	
	—Mineral Oil Tween 40/Span 80 Emulsion—						—Mineral Oil Pluronic F 68/— Span 80 Emulsion			
1.0	70.75 <sup>e</sup>		84.53		78.64		69.57		52.66	
2.5	21.28		9.70		12.39		22.06		21.60	
3.5	5.01		4.42		6.05		7.79		14.65	
4.5	1.07		0.48		0.66		0.58		3.47	
5.5	0.36		0.10		0.47				2.54	
6.5	0.36		0.19		0.85				2.16	
7.5	0.45		0.48						1.41	
8.5	0.09		0.10		0.47				0.85	
9.5					0.09				0.38	
10.5	0.09				0.38				0.28	
	100.00		100.00		100.00		100.00		100.00	
$N^f$	1,118		1,041		1,057		1,206		1,065	
$S^g$	$1.74 \times 10^4$		$1.91 \times 10^4$		$1.56 \times 10^4$		$2.35 \times 10^4$		$1.18 \times 10^4$	
$d_{av}^h$	1.57 <sup>k</sup>		1.33		1.51		1.55		2.26	
$s^i$	1.05 <sup>k</sup>		0.88		1.23		0.87		1.73	
—Peanut Oil Tween 40/Span 80 Emulsion—										
1.0	76.08		71.13		73.33		84.43		84.47	
2.5	21.45		13.06		14.30		11.19		8.94	
3.5	2.12		9.79		9.20		3.73		4.97	
4.5	0.35		2.84		2.09		0.47		0.99	
5.5			0.86		0.67		0.09		0.45	
6.5			1.37		0.25		0.09		0.09	
7.5			0.26		0.08				0.09	
8.5			0.34		0.08					
9.5			0.26							
10.5			0.09							
	100.00		100.00		100.00		100.00		100.00	
$N^f$	1,417		1,164		1,196		1,072		1,107	
$S^g$	$2.73 \times 10^4$		$1.38 \times 10^4$		$1.87 \times 10^4$		$2.58 \times 10^4$		$2.20 \times 10^4$	
$d_{av}^h$	1.39		1.73		1.57		1.29		1.32	
$s^i$	0.71		1.37		1.06		0.68		0.83	
—Safflower Oil Tween 40/Span 80 Emulsion—										
1.0	82.95		64.36		69.16		64.34		71.78	
2.5	13.10		19.45		19.53		26.99		16.57	
3.5	3.21		12.57		9.35		7.67		8.43	
4.5	0.74		2.51		1.59		0.65		1.99	
5.5			0.81		0.28		0.14		0.76	
6.5			0.30		0.09		0.07		0.47	
7.5							0.07			
8.5										
9.5										
10.5							0.07			
	100.00		100.00		100.00		100.00		100.00	
$N^f$	1,214		1,352		1,070		1,382		1,056	
$S^g$	$2.66 \times 10^4$		$1.90 \times 10^4$		$2.12 \times 10^4$		$2.09 \times 10^4$		$1.92 \times 10^4$	
$d_{av}^h$	1.33		1.75		1.60		1.64		1.59	
$s^i$	0.66		1.11		0.97		0.95		1.05	

<sup>a</sup> Oil and Span in inner funnel    <sup>b</sup> Water and Tween in outer funnel    <sup>c</sup> Oil Span and Tween in inner funnel    <sup>d</sup> Water and Pluronic in outer funnel    <sup>e</sup> Frequency expressed as per cent of  $V$     <sup>f</sup>  $N$  Total counts    <sup>g</sup>  $S$ , Specific interfacial area  
<sup>h</sup>  $d_{av}$  Arithmetic mean globule diameter    <sup>i</sup>  $s$  Standard deviation    <sup>j</sup> cm<sup>2</sup>/ml    <sup>k</sup> Microns

**Effect of Exposure Time.**—The effect of varying the insonation period of the emulsion in the Minisonic was studied. Prolonged exposure at certain frequencies will cause coarsening of the emulsion. Campbell and Long (7) and Alexander (5) reported this coagulation effect which results from collisions of dispersed particles. Higher frequencies increase the rate and energy of these collisions. The low operating frequency of the Minisonic (22 kc) would not be expected to produce coarsening of the emulsion on prolonged exposure.

**Emulsions Analyzed.**—Five 25% o/w emulsions were prepared by the premix method and were irradiated in the Minisonic homogenizer for two and one-half, five, ten, fifteen, twenty, twenty-five, and thirty minutes at a constant temperature of 35°. The emulsions were photographed and analyzed as described. The emulsions used were: (a) mineral oil with 4% Tween 40/Span 80, (b) peanut oil with 6.25% acacia, (c) peanut oil with 4% Tween 40/Span 80, (d) peanut oil with 4% Pluronic F-68/Span 80, and (e) peanut oil with 1% sodium lauryl sulfate. Figure 1 shows the effect of the various exposure periods on the five emulsions.

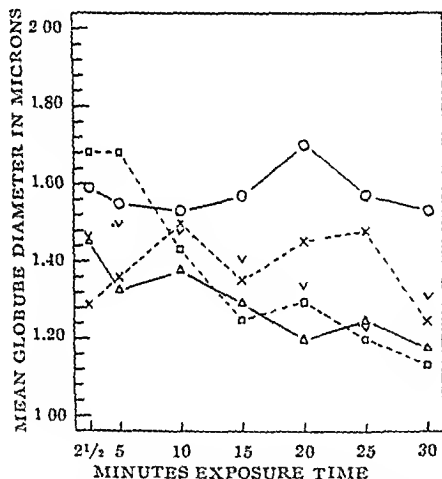


Fig. 1.—Effect on mean globule diameter of exposure to ultrasonic waves for various time intervals. Emulsions exposed: O, mineral oil-Tween 40/Span 80; □, peanut oil-acacia; Δ, peanut oil-Pluronic F-68/Span 80; ▽, peanut oil-sodium lauryl sulfate; x, peanut oil-Tween 40/Span 80.

## RESULTS

Table I shows that the method of size-frequency analysis used in this study gives a good degree of reproducibility or accuracy. Any error introduced by the analysis procedure is probably very small.

From Table II it can be seen that there was no appreciable difference in the three methods of forming the emulsions. The single-funnel premix method was at least equivalent to the two-funnel methods.

Figure 1 reveals that long insonation periods had no undesirable effects on the emulsions investigated. The low frequency of the Minisonic produced no coagulation in the emulsions. Three of the five emulsions showed decreasing mean globule diameters with increased exposure time.

Audouin and Levavasseur (8) reported that at 25 kc. o/w emulsions inverted to w/o emulsions if the exposure time exceeded fifteen minutes. If phase inversion is a characteristic of the low frequencies the emulsions of the Minisonic should have shown this phenomenon. The emulsions exposed for thirty minutes were checked by dilution and dye methods and were found to be universally oil-in-water.

## SUMMARY

A simplified method of emulsion analysis has been perfected. This method has the advantages of being rapid, accurate, and of producing a permanent record. It is easier than previous photomicrographic techniques in that pictures do not have to be printed from the negatives. Measuring visually rather than mechanically with a calibrated rule materially speeds the analysis.

The Minisonic ultrasonic homogenizer was found to operate almost equally well regardless of the method used in making the emulsion. Prolonged insonation did not cause coagulation of the emulsions. In some instances there was a continual reduction in mean globule diameter; in other cases a plateau was evident from the very beginning of the homogenization.

## REFERENCES

- (1) Harkins, W. D., and Beeman, N., *J. Am. Chem. Soc.*, **51**, 1674 (1929).
- (2) Lotzkar, H., and Maclay, W. D., *Am. Perfumer Essent. Oil Rep.*, **3**, 67 (1944).
- (3) Levius, H. F., and Drummond, F. G., *J. Pharm. and Pharmacol.*, **5**, 743 (1953).
- (4) Mullins, J. D., and Becker, C. H., *THIS JOURNAL*, **45**, 105 (1956).
- (5) Alexander, P., *Mfg. Chemist*, **22**, 5, 12 (1951).
- (6) "Atlas Guide," Atlas Powder Co., Wilmington, Del., 1956, pp. 19-26.
- (7) Campbell, H., and Long, C. A., *Pharm. J.*, **163**, 127 (1949).
- (8) Audouin, A., and Levavasseur, G., *Oléagineux*, **4**, 95 (1949).

# Emulsification With Ultrasonic Waves II\*

## Evaluation of Three Ultrasonic Generators and a Colloid Mill

By ROBERT E. SINGISER† and HAROLD M. BEAL

Three ultrasonic generators and a colloid mill were evaluated with 25 per cent mineral oil, peanut oil, and safflower oil emulsions, each made with four emulsifier systems. Emulsions were exposed in each apparatus for periods up to fifteen minutes. The best of these emulsions were analyzed after zero, fifteen, thirty, and sixty days storage. From the size-frequency analysis data specific interfacial area, mean globule diameter, and standard deviation were calculated. The Minisonic homogenizer produced emulsions distinctly superior to those made on any other apparatus.

EMULSIFICATION of vegetable and mineral oils with ultrasonic waves has progressed rapidly in the past decade and markedly so in the last several years. Prior to 1949 no extensive work was reported. Since that time significant contributions to this field have been made. Campbell and Long (1) and Audouin and Levavasseur (2, 3) experimented with emulsification of vegetable, mineral, and fish oils. The latter investigators found the ultrasonic frequency employed to be a preponderant factor in the orientation of the emulsion that formed. Beal and Skauen (4, 5) studied some of the factors influencing ultrasonic emulsification, and the effect of ultrasonic waves on various emulsifying agents.

Although many claims have been made for ultrasonic apparatus as homogenizers no comparative evaluation has been attempted. This study was undertaken to provide a measure of comparison between various ultrasonic generators and between the ultrasonic homogenizers and a conventional homogenizer. As a secondary observation the relative efficiency of the various emulsifying agents and the ease of emulsification of different oils was noted.

### APPARATUS

Four apparatus were studied; they were assigned codes as follows:

Code 1.—The Eppenbach Colloid mill, model QV-6-1, was used to represent conventional emulsifying equipment with which to compare the ultrasonic generators. The rotor-stator clearance was adjusted so that flow rate through the recirculation pipe was 700–900 ml./min. The water-cooled jacket was utilized throughout.

Code 2.—General Electric model G-3 piezoelectric-type ultrasonic generator with a quartz crystal operating at 300 kc. and a plate current of 175 ma.

\* Received August 21, 1959, from Ahhott Laboratories, Scientific Division, North Chicago, Ill.

This paper is based on a dissertation submitted by Robert E. Singiser to the Graduate School of the University of Connecticut in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Fellow, American Foundation for Pharmaceutical Education.

Irradiation of the sample was done in a 2-ounce polyethylene bottle positioned to give maximum activity in the water-cooled chamber. Sample size was limited to 20 ml.

Code 3.—Branson Ultrasonic Corp. Sonogen generator, model AP-25-B, driving at 39.1 kc. barium titanate tank-type transducer, model T-52. This piezoelectric-type generator was tuned to operate between 80–100 ma. One hundred-milliliter samples in 8-ounce polyethylene bottles were partially immersed in the water-filled tank above the transducer. Very little activity was produced and it was necessary to provide slow speed stirring to keep the emulsion components reasonably well dispersed in order that emulsification would occur.

Code 4.—Minisonic Mark 2 hydrodynamic-type ultrasonic generator manufactured by Sonic Engineering Corp. The single funnel method for using this generator (6) was employed in this study. One-liter quantities were recycled in this water-cooled apparatus.

### EXPERIMENTAL

**Emulsion Components.**—Three oils were selected for use in this investigation: heavy mineral oil U. S. P., peanut oil U. S. P., and safflower oil, edible (Pacific Vegetable Oil Corp.).

Four emulsifier systems were used in this study: (a) acacia in the same oil-gum ratio as the official acacia emulsion; (b) Tween 40<sup>1</sup>/Span 80<sup>1</sup>; (c) Pluronic F-68<sup>2</sup>/Span 80; and (d) sodium lauryl sulfate.

Distilled water containing 0.2% of the methyl and 0.02% of the propyl esters of *p*-hydroxybenzoic acid was used in the external phase of all emulsions. The safflower oil contained 0.01% propyl gallate and 0.01% citric acid as antioxidants. These antioxidants in the same concentration were also added to the aqueous phase of all safflower oil emulsions.

**Formulas of Emulsions.**—Table I shows the twelve basic 25% emulsions used in this study. Each of the three oils was prepared with each of the four emulsifier systems. The oils and emulsifier systems were coded so that easier identification of each combination was possible: M—mineral oil, P—peanut oil, S—safflower oil, A—acacia, B—Tween 40/Span 80, C—Pluronic F-68/Span 80, and D—sodium lauryl sulfate. Where possible the HLB system (7) of classifying and selecting emulsion com-

<sup>1</sup> Product of Atlas Powder Co., Wilmington, Del.

<sup>2</sup> Product of Wyandotte Chemical Co., Midland, Mich.

TABLE I—25% v/v O/W EMULSIONS STUDIED

Code	Oil	HLB	Acacia	Tween 40, <sup>a</sup> % (HLB 15.6)	Span 80, <sup>a</sup> % (HLB 4.3)	Pluronic F 68, <sup>a</sup> % (HLB 16)	Sodium Lauryl Sulfate, %	Distilled Water, <sup>b</sup> %
M-A	Mineral oil	12.0	6.25	.	.	.	.	100
M-B	Mineral oil	.	.	2.72	1.28	.	.	100
M-C	Mineral oil	.	.	.	1.36	2.64	.	100
M-D	Mineral oil	.	.	.	.	.	1.00	100
P-A	Peanut oil	8.0	6.25	.	.	.	.	100
P-B	Peanut oil	.	.	1.32	2.68	.	.	100
P-C	Peanut oil	.	.	.	2.72	1.28	.	100
P-D	Peanut oil	.	.	.	.	.	1.00	100
S-A	Safflower oil	11.5	6.25	.	.	.	.	100 <sup>c</sup>
S-B	Safflower oil	.	.	2.52	1.48	.	.	100 <sup>c</sup>
S-C	Safflower oil	.	.	.	1.52	2.48	.	100 <sup>c</sup>
S-D	Safflower oil	.	.	.	.	.	1.00	100 <sup>c</sup>

<sup>a</sup> Total mixed emulsifier concentration 4%    <sup>b</sup> Preserved with 0.2% methyl and 0.02% propyl esters of *p* hydroxybenzoic acid    <sup>c</sup> Antioxidants added: 0.01% propyl gallate and 0.01% citric acid

ponents was used in formulating the emulsions. HLB values for Pluronic F-68 and safflower oil were experimentally determined, the other HLB values were obtained from the literature (7, 8). Table I lists these values.

Premixes of each of the twelve basic emulsions were prepared by shaking in a gallon bottle. This permitted uniform aliquots to be withdrawn and emulsified in the various apparatus. The orientation of each of the emulsions was confirmed to be o/w by the addition of dyes and by dilution techniques.

**Evaluation.**—Each of the twelve emulsions was insonated in each of the four apparatus for two and one-half, five, ten, and fifteen minutes. Each emulsion thus formed was evaluated by the photomicrographic size-frequency method previously described (6). A minimum of 1,000 globules was measured for each sample. The best emulsion from the four insonation periods was then stored at room temperature and analyzed again at fifteen, thirty, and sixty days.

A 50% propylene glycol solution was used as the diluting fluid for all emulsions except those made with acacia. The acacia-stabilized emulsions broke when diluted with propylene glycol so a 12.5% acacia solution was used. Both fluids effectively prevented Brownian movement and have been previously reported as satisfactory diluents (4, 9, 10).

**Code.**—The code identifies the emulsion as to the oil used (M-P-S), the emulsifier system (A-B-C-D), and the homogenizer (1-2-3-4). A typical code reads M-A-4, indicating a mineral oil emulsion (M) with acacia (A) as the emulsifier, the emulsion being made on the Minisonic (code 4) homogenizer.

**Parameters.**—The specific interfacial area and the arithmetic mean diameter with standard deviation were calculated from the analysis data. The specific interfacial area (*S*) was calculated following the method of Mullins and Becker (8). While this value is often reported alone it is difficult to visualize and gives very little indication of the type of distribution it represents. The inclusion or exclusion of one globule in the smaller class intervals has little effect on the specific interfacial area while in the larger class intervals this one globule changes the value considerably. The mean globule diameter (*d<sub>ar</sub>*) is not subject to such weighted influence of

one globule. The mean globule diameter with standard deviation (*s*) gives a good indication of the type of distribution in a sample. The standard deviation has been used by Jellinek (11) who described it as the inhomogeneity factor. A large value indicates much variance in the distribution and a small value indicates high uniformity.

Complete data on all of the emulsions analyzed cannot be given here. The mean globule diameter was selected as representative of the results obtained. These data for the stored emulsions are graphed as follows: Figures 1-4 illustrate the mineral oil emulsions, Figs. 5-8 the peanut oil emulsions, and Figs. 9-12 the safflower oil emulsions.

DISCUSSION

Due to crystal fracture on the General Electric unit some emulsions could not be made on it. The unit's small capacity precludes any commercial use of this generator as a homogenizer. Beal and Skauen (4, 5) have done extensive work with this unit.

Where graphs show only a single "0 days" entry for certain emulsions, free oil was found at the fifteen-day observation. No further analysis was made when this gross instability was observed.

**Apparatus.**—Figures 1-12 indicate that the Minisonic homogenizer produces emulsions superior to those produced on the other apparatus. Generally, the emulsions made on the Minisonic had a smaller mean globule diameter (or larger specific interfacial area) than any others. These emulsions also had greater uniformity. With the exception of the mineral oil emulsions, the Minisonic-made emulsions showed very few particles larger than 5.5  $\mu$ . This degree of uniformity was not found in any other apparatus. The Eppenbach mill, the General Electric, and Sonogen generators were approximately equivalent in the degree of dispersion produced.

Of the apparatus evaluated only the Eppenbach mill and the Minisonic generator are adaptable for production use. It would be expected that the fifteen-minute exposure samples from the Eppenbach mill would be best. The rotor-stator arrangement simply provides shear for disrupting the oil phase. In three instances (M-A, M-D, S-A) samples other than the fifteen-minute exposure samples were found to be best. Of all the units the Eppen-



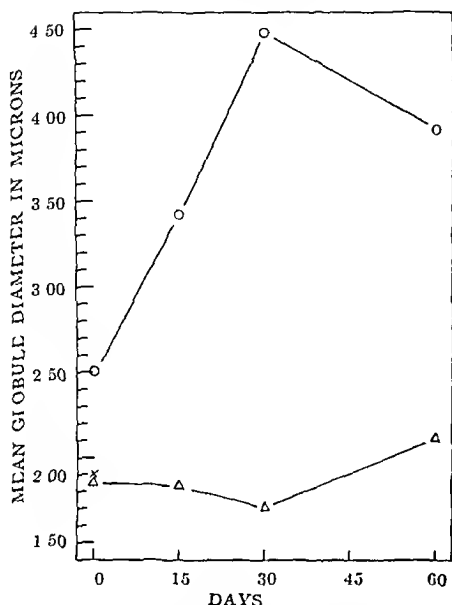


Fig 1—Mean globule diameters of stored mineral oil/aceia emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; Δ, Minisomic ultrasonic generator; x, Sonogen ultrasonic generator.

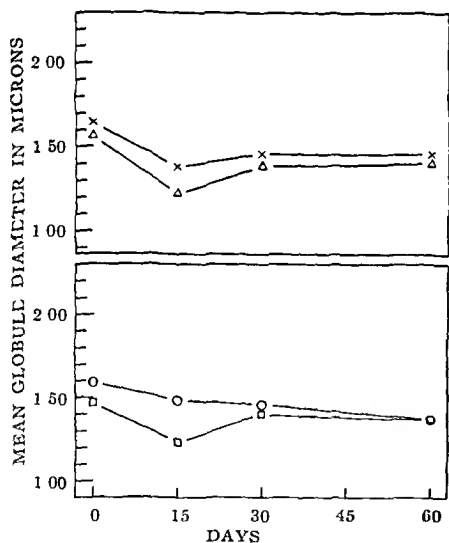


Fig 2—Mean globule diameters of stored mineral oil/Tween 40/Span 80 emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; □, G E ultrasonic generator; Δ, Minisomic ultrasonic generator; x, Sonogen ultrasonic generator.

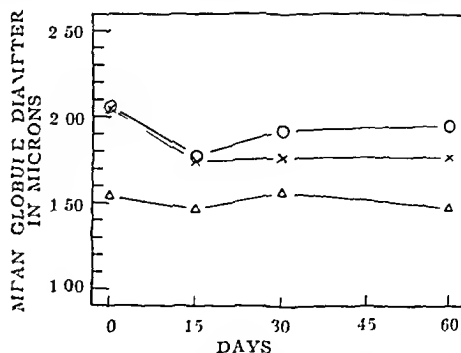


Fig 3—Mean globule diameters of stored mineral oil-Pluronic F-68/Span 80 emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; x, Sonogen ultrasonic generator; Δ, Minisomic ultrasonic generator.

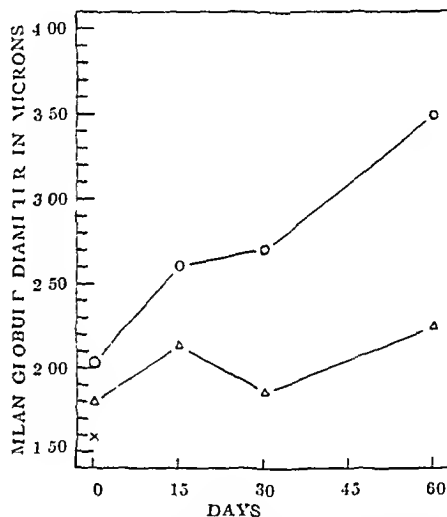


Fig 4—Mean globule diameters of stored mineral oil-sodium lauryl sulfate emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; x, Sonogen ultrasonic generator; Δ, Minisomic ultrasonic generator.

bach mill had the greatest tendency to incorporate air into the emulsion. It is recognized that air entrapment in emulsions may accelerate their breakdown (12). This may be the reason for the inferior long exposure samples.

Table II summarizes the mean globule diameters and average specific interfacial area produced by the different apparatus. These figures are the

average of the four stability storage results. Since few of the emulsions underwent any marked deterioration during the sixty days storage, this average value is useful for comparisons, but it has no value beyond that. This lack of change in the emulsions undoubtedly parallels the findings of Harkins and Beeman (13) who noted that when sufficient emulsifying agents were used to complete a monomolecular layer about the oil droplets, the emulsion could remain appreciably unchanged for as long as one year. King and Mukherjee (14) found vast stability differences by changing emulsifier concentration.

The Eppenbach mill had an aggregate average particle diameter of  $2.36 \mu$ , the Minisomic generator produced globules averaging  $1.48 \mu$ . These average values represent measurements on approximately 55,000 globules.

The 300 kc frequency of the General Electric

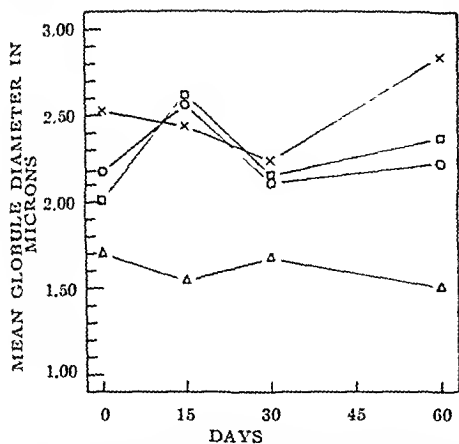


Fig. 5.—Mean globule diameters of stored peanut oil-acacia emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

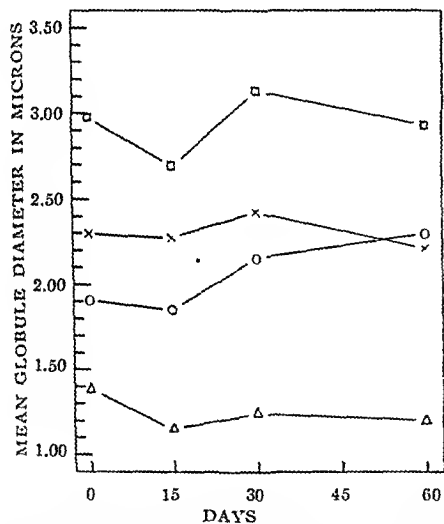


Fig. 6.—Mean globule diameters of stored peanut oil-Tween 40/Span 80 emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

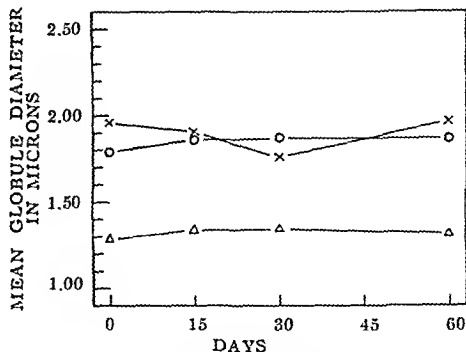


Fig. 7.—Mean globule diameters of stored peanut oil-Pluronic F-68/Span 80 emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

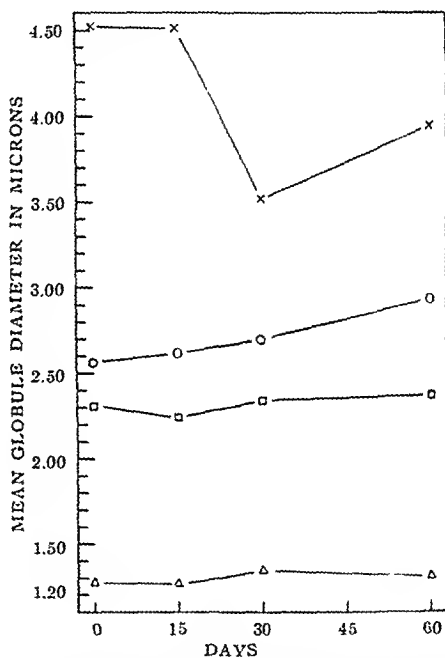


Fig. 8.—Mean globule diameters of stored peanut oil-sodium lauryl sulfate emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

unit has a marked tendency to cause coagulation of the emulsion on prolonged exposure. In all cases the ten-minute exposure samples were better than the fifteen minute ones. Using the same generator Beal and Skauen (5) found a gradual improvement in their emulsions up to thirty minutes. In this study a polyethylene bottle was used as the exposure chamber. Polyethylene has poor heat transfer qualities. Even though the bottle was immersed in a cooling bath, temperatures of 55–60° developed in the emulsion on fifteen minutes insonation. Bondy and Sollner (15), Audouin and Levavasseur (2), and others (16) have noted that elevation of temperature does not favor emulsification, but actually favors coagulation.

The only emulsions to show separation of free oil were those prepared in the Sonogen generator. The Sonogen is not well suited for the production of emulsions. The ultrasonic waves produce very little agitation in the exposure tank, and as a result some type of stirring must be provided to keep the emulsion components in intimate contact. In this study minimal stirring was employed. Even so, it is questionable how much of the emulsification is attributable to stirring and how much is actually due to ultrasonic cavitation. In addition, there is no way to maintain a constant temperature with this apparatus, which is designed mostly as a cleaning tool.

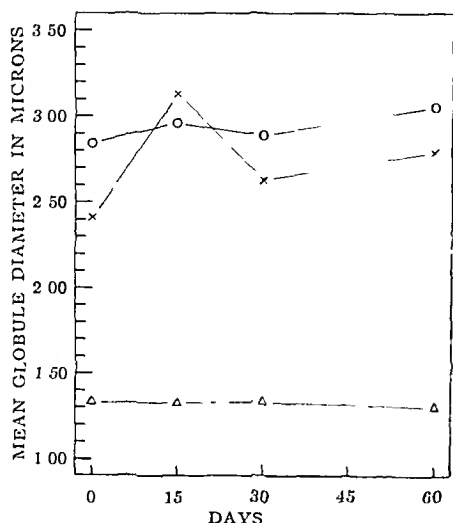


Fig. 9.—Mean globule diameters of stored saf-flower oil-acacia emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

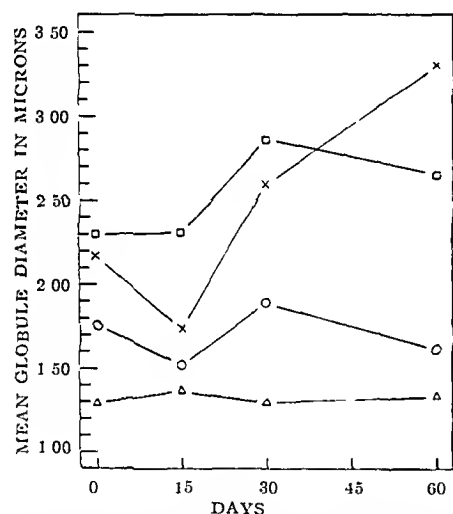


Fig. 10.—Mean globule diameters of stored saf-flower oil-Tween 40/Span 80 emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

**Emulsions.**—Sodium lauryl sulfate-stabilized emulsions showed the greatest tendency for instability, especially when prepared in the Eppenbach colloid mill (M-D-1, M-D-4, P-D-1, S-D-1). These emulsions have a very low viscosity which more than likely contributes to their instability. King and Mukherjee (14) found a high viscosity essential to good stability.

Table II shows that the nonionic emulsifier pairs, Tween 40/Span 80 (series B) and Pluronic F-68/Span 80 (series C), were generally the best of the agents used. Neither of these emulsion systems showed oil separation. Siragusa, *et al.* (17), found

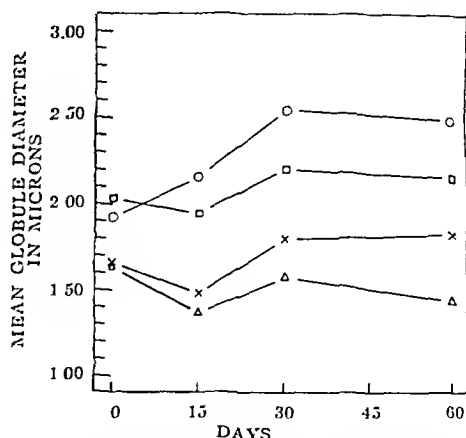


Fig. 11.—Mean globule diameters of stored saf-flower oil-Pluronic F-68/Span 80 emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; □, G. E. ultrasonic generator; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

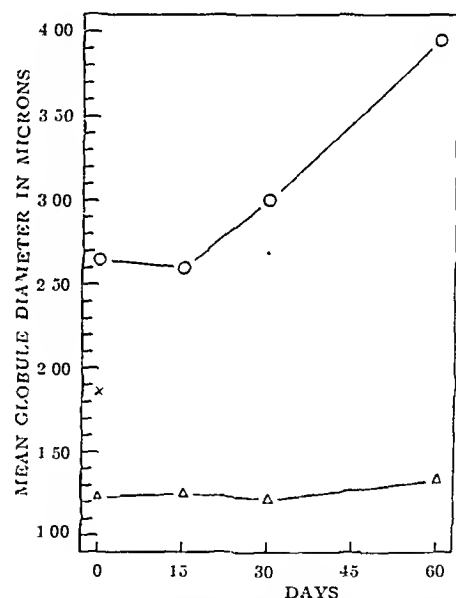


Fig. 12.—Mean globule diameters of stored saf-flower oil-sodium lauryl sulfate emulsions. Emulsifying apparatus: O, Eppenbach colloid mill; x, Sonogen ultrasonic generator; Δ, Minisonic ultrasonic generator.

a Tween 80/Span 20 emulsifier combination better for a 12.5% mineral oil emulsion than acacia, both made in the Eppenbach mill. King and Mukherjee noted that the efficiency of an emulsifying agent is a very specific property. Mixed emulsifiers should give better emulsions than the single agents since it is possible to match the emulsifier HLB with the HLB required by the oil.

According to the work of Chun, *et al.* (18), the HLB value of acacia is 8.0. Since the HLB requirement of peanut oil is also 8.0 for an o/w emulsion

TABLE II.—SUMMARY OF DATA ON EMULSIONS<sup>a</sup>

Emul- sion	Eppenbach			General Electric			Sonogen			Minisonic		
	<i>dar</i> <sup>b</sup>	<i>s</i> <sup>c</sup>	<i>Sd</i>	<i>dar</i>	<i>s</i>	<i>S</i>	<i>dar</i>	<i>s</i>	<i>S</i>	<i>dar</i>	<i>s</i>	<i>S</i>
M-A	3.61	3.12	0.74	e	e	e	f	f	f	1.88	1.30	1.58
M-B	1.48	1.13	1.43	1.37	0.88	1.95	1.49	1.04	1.66	1.40	1.00	1.64
M-C	1.93	1.04	2.03	e	e	e	1.84	0.98	2.13	1.52	0.84	2.41
M-D	2.68	2.74	0.75	e	e	e	f	f	f	2.02	1.69	1.20
P-A	2.26	1.80	1.12	2.28	1.80	1.06	2.48	2.35	0.88	1.61	0.83	2.46
P-B	2.05	1.82	0.99	2.92	2.25	0.94	2.30	1.50	1.35	1.26	0.60	3.11
P-C	1.85	1.49	1.26	e	e	e	1.90	1.78	1.03	1.33	0.76	2.44
P-D	2.71	2.47	0.84	2.31	2.14	0.91	4.06	3.12	0.72	1.30	0.61	3.09
S-A	2.94	2.56	0.83	e	e	e	2.75	2.58	0.82	1.33	0.66	2.91
S-B	1.70	1.40	1.24	2.52	1.77	1.13	2.45	2.60	0.78	1.33	0.70	2.68
S-C	2.27	2.01	1.02	2.08	1.68	1.21	1.69	1.34	1.41	1.51	0.84	2.40
S-D	3.06	2.54	0.88	e	e	e	f	f	f	1.27	0.59	3.15

<sup>a</sup> Average of zero-, fifteen-, thirty-, and sixty-day determinations<sup>b</sup> *d<sub>m</sub>*, Arithmetic mean globule diameter,  $\mu$ .<sup>c</sup> *s*, Standard deviation:  $s = \sqrt{\frac{n(\sum f s^2) - (\sum f s)^2}{n(n-1)}}$ ,  $\mu$ .<sup>d</sup> *S*, Specific interfacial area,  $\times 10^4$  cm<sup>2</sup>/ml.<sup>e</sup> Generator failure, no emulsion prepared<sup>f</sup> Oil separation at fifteen days.

it would be anticipated that the peanut oil-acacia (P-A) emulsions would be superior to mineral oil-acacia (M-A) or safflower oil-acacia (S-A) emulsions. Table II shows that this is generally true. The mean globule size of all P-A emulsions is 2.13  $\mu$ , 2.67  $\mu$  for M-A emulsions, and 2.32  $\mu$  for S-A emulsions. The acacia emulsions were uniformly coarser than the Tween 40/Span 80 or Pluronic F-68/Span 80 emulsions. King and Mukherjee (14) found emulsions stabilized by hydrophilic colloids to be generally coarse, but usually stable, at least in part due to their high viscosity.

From the data it appears that all three of the oils have approximately the same ease of emulsification when handled as they were in this investigation.

In diluting the emulsions for photomicrography a preliminary indication of the better emulsions was observed. Generally dilutions of 1:100 to 1:200 produced fields suitable for measurement. In emulsions prepared in the Minisonic it was necessary to dilute to 1:450 to get few enough particles to analyze satisfactorily. Even at this dilution there were many more globules in the field than found with the other emulsions. Emulsions other than the Minisonic ones broke when such extreme dilutions were made. The counting method used by Beal and Skauen (4, 5) where exact

dilutions are made would undoubtedly have shown the Minisonic emulsions to be even more superior than the evaluation methods of this study showed.

## REFERENCES

- (1) Campbell, H., and Long, C. A., *Pharm. J.*, 163, 127(1949)
- (2) Audouin, A., and Levavasseur, G., *Oléagineux*, 4, 95(1949)
- (3) *Ibid*, *Mem. serv. chim. Etat Paris*, 39, 213 (1954)
- (4) Beal, H. M., and Skauen, D. M., *THIS JOURNAL*, 44, 487(1955).
- (5) *Ibid*, 44, 490(1955).
- (6) Singiser, R. E., and Beal, H. M., *ibid.*, 49, 478(1960)
- (7) "Atlas Guide," Atlas Powder Co., Wilmington, Del., 1956, pp 19-26.
- (8) Mullins, J. D., and Becker, C. H., *THIS JOURNAL*, 45, 105(1956).
- (9) Levius, H. P., and Drummond, F. G., *J. Pharm. and Pharmacol.*, 5, 743(1953).
- (10) Smith, E. L., and Grinling, G. N., *Quart. J. Pharm. and Pharmacol.*, 3, 354(1930).
- (11) Jellinek, H. H. O., *J. Soc. Chem. Ind.*, 69, 225(1950)
- (12) Chen, J. L., Cyr, G. N., and Langlykke, A. F., *Drug & Cosmetic Ind.*, 81, 596(1957).
- (13) Harkins, W. D., and Beeman, N., *J. Am. Chem. Soc.*, 51, 1674(1929)
- (14) King, A., and Mukherjee, L. N., *J. Soc. Chem. Ind.*, 58, 243(1939)
- (15) Bondy, C., and Sollner, K., *Trans. Faraday Soc.*, 32, 556(1936)
- (16) Arbussa, J. M. S., *Galenica Acta Madrid*, 9, 195 (1956), through *Chem. Abstr.*, 51, 17105d(1957).
- (17) Siragusa, J. M., Husa, W. J., and Becker, C. H., *Am. J. Pharm.*, 129, 152(1957).
- (18) Chun, A. C. H., Joslin, R. S., and Martin, A. N., *Drug & Cosmetic Ind.*, 82, 164, 312(1958).

## Book Notices

*Einführung in die Pharmokopsychologie* By HERBERT LIPPERT Band 4 der Enzyklopadie der Psychologie in Einzeldarstellungen Verlag Hans Huber, Bern and Stuttgart, 1959 Intercontinental Medical Book Corp., 381 Fourth Ave., New York 16, N Y 254 pp 15 X 23 cm Price Fr /DM 32

This book (in German) is intended as an introductory textbook in pharmacopsychology. The final chapter covers the relation of chemical structure and psychic action of barbiturates, phenyl aminoethane derivatives, opium alkaloids, and synthetics, cocaine atropine group, alcohol ether group, and ataractic tranquilizers.

*Recent Advances in Neuro Physiological Research* Edited by D EWEN CAMERON and MILTON GREENBLATT American Psychiatric Association, 1700 Eighteenth St., N W, Washington 9, D C, 1959 136 pp 15 X 23 cm Paperbound

This is a compilation of nine reports on recent research in this field covering communication, memory disturbance, electrophysiology, animal behavior, and a biological basis for psychopathology. Discussions of the papers are included, but there is no index.

*The United States Pharmacopeia* 16th rev. Published by the U S Pharmacopeial Convention, Inc., 1960 Distributed by Mack Publishing Co., Easton, Pa 414 + 1148 pp 15 X 23 cm Price \$10 domestic, \$10.50 foreign

The U S P revision committees have maintained such a high degree of excellence for so long that when a new revision of the compendium appears we are inclined to accept it as something automatically produced. U S P XVI unquestionably reflects the competent medical and pharmaceutical efforts of a dedicated group of experts in these fields. The 908 monographs in U S P XVI include 225 new admissions, which represent every new class of drugs introduced since 1955. These include the oral antidiabetics, orally effective diuretics, antihypertensives, and anticarcinogenic agents. In addition there are listed 81 titles of "U S P XVI interim admissions." Monographs for these articles, which have been approved for admission by the U S P Committee on Scope, are still being developed and should appear in Interim Revision Announcements or in Supplements to U S P XVI. Progressive analytical studies are reflected in the applications of new analytical techniques such as infrared spectrophotometry, chromatography, radioisotope tracers, etc., to drugs in retained monographs as well as in new monographs. The increase in spectrophotometric procedures for identification tests and assays is reflected in the addition of 78 new U S P Reference Standards, most of them being required for spectrophotometric methods.

The style and format of the monograph section of the book is similar to that in U S P XV but the see

tion headed "General Tests, Processes and Apparatus" is set in space saving two column format. This section covers 272 pages and has its separate table of contents. On behalf of the U S P revision committee and those who assisted in the preparation of U S P XVI, chairman Lloyd C. Miller and his executive assistant Adley B. Nichols should receive our commendation. The new official compendia, U S P XVI and N F XI, the technical revision of which is attributable mainly to pharmacy members of the revision committees, are splendid proof that pharmacy can set its standards high in the professional aspects of its practice.

*Optical Rotatory Dispersion* By CARL DJERASSI McGraw Hill Book Co., 330 West 42nd St., New York 36, N Y 1960 411 + 293 pp 15 X 23 cm

This book deals with rotatory dispersion as a physical tool used to solve structural, stereochemical, and conformational problems in organic chemistry and biochemistry. It considers mainly the advances made since 1955 with particular emphasis on steroids, terpenes, amino acids, and proteins and peptides. The increased interest and experimental work in the field has been greatly stimulated by the acquisition of spectropolarimeters by many laboratories. Three of the 17 chapters have been written by experts in their fields of instrumentation and application of optical rotatory dispersion. Appendixes include a bibliography of publications, an explanation of the nomenclature, and stereochemical conventions for steroids and triterpenoids. Author and subject indexes are given.

*Stress and Cellular Function* By H. LABORIT, et al J. B. Lippincott Co., Medical Dept., East Washington Square, Philadelphia 5, Pa., 1959 41 + 255 pp 15.5 X 23 cm Price \$7.50

This book, intended for the clinician, is a translation of the original French edition which had the title "Resuscitation: Physiological Bases and General Principles." The author attempts to outline three basic concepts from which he proposes to develop a series. He states: "Knowledge of systemic reaction to injury under its diversified aspects, will lead to a particular understanding of its management as well as of resuscitation." Resuscitation is the term used to indicate the restoration to normal values found in the healthy body.

*Technique et Pratique de la Correction Prothétique des Surdités* By JACQUES DEHAUSSY Masson et Cie., Editeurs, 120, boulevard Saint Germain, Paris 6<sup>e</sup>, France, 1960 265 pp 17 X 25 cm Paperbound Price NF 30,000

This book (in French) includes methods of testing hearing ability, diagnosing abnormalities, and describes corrective procedures. It is directed to the specialist in this branch of medical practice.

*Scientific Edition*

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49

AUGUST 1960

NUMBER 8

## Infrared Analysis of Pharmaceuticals I\*

### Application of the Potassium Bromide Disk Technique to Some Steroids, Alkaloids, Barbiturates, and Other Drugs

By ALMA L. HAYDEN and OSCAR R. SAMMUL

The quantitative potassium bromide disk technique has been investigated and methods have been adopted for its application to some pharmaceuticals. Average deviations from linearity within  $\pm 4.0$  per cent were obtained with some crystalline compounds. Analyses of pharmaceutical preparations, by hand-grinding or vibrator-grinding methods, resulted in average agreement within  $\pm 3$  to  $\pm 4.5$  per cent with ultraviolet spectrophotometric or gravimetric determinations.

THE PUBLICATION in 1952 (23, 26) of the potassium bromide disk method has resulted in investigations into the applicability of this method to compounds of varied origin. There have been reports of successful applications, both qualitatively and quantitatively, to steroids (9, 12, 20, 21), carbohydrates (2, 28), amino acids and peptides (7, 24, 26), pesticides (27), and other organic and inorganic compounds (4-6, 11, 14, 29). From these studies, it has been shown that this method is useful in the study of water-soluble materials and of fractional milligram amounts of substances difficultly soluble in the usable infrared transparent solvents. Another advantage is that some substances which are structurally similar and whose spectra in solution are essentially identical may be differentiated by infrared spectra of their potassium bromide disks (10).

In this laboratory, there developed a need for a method of determining and identifying fractional milligram amounts of pharmaceuticals, some of which were difficultly soluble in the usable infrared solvents. In view of the reports of success with quantitative potassium bromide methods (5, 12, 14, 17, 21, 24, 25, 27, 29), and with an awareness of the possible formation of anomalous spectra (1-3, 8, 10, 18-20), an investigation was made of the applicability of a quantitative potassium bromide method to some pharmaceuticals.

Several methods have been used in obtaining homogeneous potassium bromide-compound mixtures. Kirkland (14) studied various methods of dispersing samples in potassium bromide and concluded that vibrator-grinding gave the most reproducible mixtures. Wiberley and co-workers (29) used vibrator-grinding of potassium bromide-sample with potassium thiocyanate as an internal standard in quantitative studies of poly(vinyl chloride)-poly(vinyl acetate) copolymers. Schwarz and co-workers (25) used lyophilization to obtain homogeneous mixtures of potassium bromide and desoxyribonucleic acid. Rosenkrantz and co-workers (21) estimated to within 8 per cent of the known amount for steroids mixed by spatula with potassium bromide. Susi and Reetor (27) used vibrator-grinding of crystalline materials in the quantitative determination of mixtures of pesticides. These authors reported 5 per cent deviations from the Beer-Lambert law

\* Received October 30, 1959, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Washington 25, D. C.

The authors are greatly indebted to Mr. Jonas Carol for his advice and criticism throughout this work.

for standard pesticides. Itô and Amakasu (12) found that trituration in a mortar provided satisfactory preparation of ethinyl estradiol samples, which could be analyzed with an average accuracy of about 2 per cent. Ingebrigtsen and Smith (11) found that the mixtures prepared by hand-grinding a slurry of the sample and potassium bromide in a volatile solvent gave spectra of excellent quality with increased resolution and absorbance.

In the work reported here, the authors made a preliminary investigation into various methods of dispersing the sample in potassium bromide. It was found that hand-grinding of solutions of compounds with potassium bromide and vibrator-grinding of residues from solution or of recrystallized samples with potassium bromide without an internal standard gave the most reproducible homogeneous mixtures which could be studied quantitatively. Since the crystalline forms of some compounds depend on the solvent and the conditions of crystallization, the standard and sample solutions were subjected to identical treatment.

Some standard steroids, alkaloids, barbiturates, and other compounds were found to agree within  $\pm 1.0$ – $4.0$  per cent with the Beer-Lambert law over the concentration range of 0.05–0.40 per cent by weight. The results with some cinchona alkaloids are given elsewhere (10).

Application of the described procedures to quantitatively prepared (synthetic) mixtures, to recovery experiments, and to drugs containing hydrocortisone and 17-hydroxy-11-desoxycorticosterone, ethinyl testosterone, phenobarbital, or acetophenetidin and caffeine revealed an average agreement within 3.0 per cent of the added amount or of results obtained by ultraviolet spectrophotometry and within 4.5 per cent of gravimetric measurements.

## EXPERIMENTAL

The potassium bromide (Harshaw 200/325) was dried at  $105^\circ$  for a minimum of sixteen hours prior to use. The 20/40 mesh material was prepared in this laboratory from Harshaw random size potassium bromide and was dried as above.

All of the substances studied were purified by recrystallization from a suitable solvent, and their melting points were determined on a Fisher-Johns melting block. For the analytical studies, solvents were chosen from which each of the compounds crystallized without glassy or amorphous material, and solutions of 1.0 mg. per ml. were used.

### Methods

**Preliminary Sample Dispersion Experiments.**—Initial experiments with potassium thiocyanate as an

internal standard used lyophilization and hand-grinding as the means of producing the mixtures. For the lyophilization procedure, 5 ml. of a 4% aqueous solution of potassium bromide containing 0.1% potassium thiocyanate was frozen with about 0.5 ml. alcoholic solutions of ethinyl estradiol, hydrocortisone, cortisone, or reserpine while being mechanically rotated. The frozen material was then dried under high vacuum. The dried mixture was mixed briefly with a spatula and a 200-mg. aliquot pressed into a disk. When hand-grinding was employed, an aliquot of a solution containing the sample was ground with 200 mg. of a 0.1% solid mixture of potassium thiocyanate in 200-mesh potassium bromide.

Some early attempts were made to disperse residues from solution with potassium bromide by evaporating the solution to dryness on potassium bromide powder, or by evaporating the solution to dryness in a mortar with subsequent addition of potassium bromide and grinding. When vibrator-grinding of crystalline samples was attempted, it was necessary to weigh small amounts, less than 1 mg., on a microbalance.

**Final Hand-Grinding Procedure.**—An aliquot of a solution (1.0 mg. per ml.) containing 0.05–1.0 mg. of the compound was ground for an accurately measured and reproduced time (five to ten minutes) with 200 mg. of potassium bromide in a 50-mm. mullite mortar with pestle. The mixture was freed of last traces of solvent and adsorbed water in vacuum at room temperature or at a temperature above the boiling point of the solvent.

**Final Vibrator-Grinding Procedure.**—A Crescent amalgamator fitted with a steel capsule ( $\frac{5}{16}$ -inch inside diameter, 1 inch in length) containing three steel balls ( $\frac{1}{8}$  inch in diameter) was used for vibrator grinding. An aliquot of a solution containing 0.05–2.0 mg. of the compound was evaporated to dryness with dry nitrogen in the steel capsules containing the steel balls. Standard and sample solutions were evaporated under the same conditions at the same time. The residue was dried at room temperature in vacuum over  $P_2O_5$  for about one hour, 400 mg. of potassium bromide was added, and the cylinder stoppered. The contents were vibrated for a predetermined optimum time (five to twenty minutes).

**Disk Pressing Technique.**—In general, the potassium bromide mixtures and disks were prepared at room temperature at less than 50% relative humidity to minimize the adsorption of water. Under conditions of greater than 50% relative humidity, the mixtures were dried at  $105^\circ$  at atmospheric pressure, or at an equivalent temperature under high vacuum. The effects of adsorbed water can be reduced by grinding at temperatures higher than atmospheric temperature. For each disk, 200 mg. of potassium bromide-compound mixture was pressed in a Beckman evacuable die which forms disks of 12.7 mm. in diameter and about 0.57 mm. in thickness. After evacuation at less than 1 mm. Hg for one minute, a force of 20,000–25,000 pounds was applied for three minutes during evacuation. After pressing, the disks were placed in vials, heated at  $105^\circ$  for ten to twenty minutes, and cooled to room temperature in a desiccator.

**Measurement of Spectra.**—The spectra were ob-

tained with a Perkin-Elmer Model 21 double-beam spectrophotometer with sodium chloride optics. The disks were inserted in the sample beam by means of a disk holder which fitted the microcell adapter. The qualitative or quantitative measurements were made without compensation in the reference beam.

A preliminary qualitative spectrum of the 2-15 $\mu$  region was made of a disk of each compound (about 0.25-0.50%, by weight) which was prepared under moderate hand- or vibrator-grinding conditions (one or two minutes grinding). This spectrum served as a guide in the selection of bands for quantitative study and in the detection of spectral variations.

For quantitative work, the baseline procedure was used to obtain the absorbance using minima on each side of the chosen maximum. After the first absorbance measurement, the disk was turned over in the holder and a second measurement was made. The average was taken of the two baseline absorbance readings. The disk area exposed to the beam was outlined at the time of the first reading and the average thickness was obtained by making six readings of this area with a micrometer. The product of the average thickness and the per cent concentration was considered the effective concentration in the sample beam. A plot of baseline absorbance against effective concentration for a compound revealed the agreement with the Beer-Lambert law over the concentration range studied. For some compounds, two or three bands were chosen from the qualitative spectra and the average of results from these bands was computed.

For a determination of deviation from the Beer-Lambert law, it was necessary to determine the optimum grinding time conditions. Since preliminary work showed that most of these compounds follow the Beer-Lambert law at a concentration of 0.25%, mixtures containing 0.25% of compound were ground for times varying between one and twenty-five minutes by one of the given procedures. By plotting an absorptivity coefficient (baseline absorbance/thickness  $\times$  concentration) against grinding time for the selected band (s), and by observing the spectrum at the various grinding times, the optimum grinding time was ascertained. For most compounds ten minutes hand-grinding was optimum. The optimum vibration-time varied with the compound, the size of the potassium bromide matrix, and the number of steel balls used.

Using the optimum grinding time, calibration curves showing the relationship between baseline absorbance and effective concentration were made by studying disks of different concentrations at the chosen wavelengths. A concentration which fell on the linear portion of the curve was chosen for analytical purposes.

Analyses of quantitatively prepared (synthetic) mixtures preceded recovery experiments where the standard compounds were used in the various separation procedures given below. The amounts determined by the infrared method were compared with the amounts added or with the amounts calculated from ultraviolet or gravimetric measurements. For most of the experiments, the averages of analyses on duplicate disks were used. The identities of the analyzed compounds were proven by comparison of the spectra of sample and standard disks of 0.10-0.25% by weight.

## Analyses of Various Pharmaceutical Preparations

**Ethinyl Testosterone.**—A weighed amount of ethinyl testosterone tablet mixture equivalent to 25 mg. of active ingredient was chromatographed on a Celite-water column (10 Gm.-5 ml.). The column was washed with 50 ml. isooctane and the excess isooctane blown out with gentle air pressure. The ethinyl testosterone was eluted with 150 ml. chloroform and the eluate evaporated to dryness in vacuum at less than 50° or with a stream of dry air at less than 40°. The residue was dissolved in absolute methanol and diluted to volume in a 25-ml. volumetric flask.

Aliquots (0.10 ml.) of methanolic solutions of the sample and of the standard (1.0 mg. per ml.) were mixed with 200 mg. potassium bromide (200 mesh or 20/40 mesh) by the hand-grinding (ten minutes) or vibrator-grinding (six minutes) procedures. For determinations using the 9.44 $\mu$  band, aliquots of 0.8 ml. were required. Baseline absorbance measurements were made of the  $\text{—C=C—C=O}$  band at 6.03 $\mu$ . The absorptivity coefficient,  $K_{std}$ , was calculated for the standard from the equation  $K_{std} = A_b/CL$ , where  $A_b$  is baseline absorbance,  $C$  is concentration in per cent by weight, and  $L$  is average thickness in millimeters. From the following equation, the calculation of amount of active ingredient in the sample tablet was made.

$$\frac{A_b}{K_{std} \times L_{sample}} \times \text{wt. KBr mixture} \times \frac{\text{Total vol.}}{\text{Aliq. vol.}} \times \frac{\text{Av. wt. per tablet}}{\text{wt. of sample}} = \text{mg. per tablet.}$$

**Hydrocortisone.**—A quantity of tablet mixture equivalent to 20 mg. of hydrocortisone was suspended in dilute acid solution and completely extracted with chloroform. The combined extracts were evaporated to dryness, and the residue was chromatographed on a Celite-formamide-water column. The column was eluted successively with benzene and with chloroform. The benzene and the chloroform eluates were washed with water and were dried with anhydrous sodium sulfate. The dried eluates were filtered, evaporated to dryness, and the residues made to volume with absolute methanol. Aliquots of the methanolic solutions were analyzed using the hand-grinding procedure. The resulting potassium bromide disks were compared quantitatively and qualitatively with standard disks of 17-hydroxy-11-desoxycorticosterone (0.15% by weight) and of hydrocortisone (0.25% by weight) which were prepared at the same time from methanolic solutions of the standard compounds. The quantitative determinations were made of the  $\text{—C=C—C=O}$  band for each compound (5.99 $\mu$  17-hydroxy-11-desoxycorticosterone, 6.05 $\mu$  hydrocortisone).

**Phenobarbital.**—A weighed amount of phenobarbital tablet mixture equivalent to 50 mg. active ingredient was dissolved in 5 ml. (2 + 1) formamide-water, and mixed with 5 Gm. acid-washed Celite. This mixture was chromatographed on a modified Sabatino (22) Celite-formamide and water column [5 Gm.-5 ml. (2 + 1)]. A base layer of 1 Gm. acid-washed Celite mixed with 1 ml. saturated barium hydroxide solution was used to remove any stearates



present in the tablet.<sup>1</sup> After a preliminary elution with 150 ml. iso-octane-chloroform (1 + 1), phenobarbital was eluted with 200 ml. water-washed chloroform. The chloroform eluate was washed with water, diluted with approximately 2% ethyl alcohol by volume, and evaporated to dryness with dry air at less than 60°. The residue was dried in vacuum over  $P_2O_5$  for thirty minutes, and was made to volume in benzene in a 50-ml. volumetric flask. Aliquots (0.4 ml.) of the sample solution and of a standard solution (1.0 mg. per ml.) were made into disks (0.1% by weight) using the hand-grinding procedure. The quantitative determinations were made using the carbonyl band at 5.88 $\mu$ .

**Phenobarbital in the Presence of Amino-phylline.**—A weighed portion of sample equivalent to about 50 mg of phenobarbital was suspended in 50 ml HCl (1 + 1). The mixture was completely extracted with ether. The combined ether extracts were washed with two 10-ml. portions of HCl (1 + 1), and with water, and were evaporated to near dryness with dry air at less than 35°. The residue was treated as described under "Phenobarbital."

**Acetophenetidin and Caffeine in APC Tablets.**—The acid-base column described by Levine (15) was modified by using two and one-half times the amounts of column components and twice the amounts of active ingredients, and by incorporating a wash layer consisting of 5 Gm. Celite and 5 ml. distilled water at the top of the column.

An amount of tablet mixture containing about 7.4 mg. acetophenetidin, 1.4 mg. caffeine, and 10 mg. aspirin was chromatographed on the modified column. The acetophenetidin was eluted with ether, the eluate was evaporated to dryness, and made to volume (5 ml.) in absolute methanol. The caffeine was eluted with water-washed chloroform, the eluate was diluted with 5% ethyl alcohol by volume, evaporated to dryness, and made to volume (2 ml.) in benzene. The aspirin was not determined.

Solutions of standard acetophenetidin in methanol (1.48 mg. per ml.) and of standard caffeine in benzene (0.72 mg. per ml.) were prepared from accurately weighed crystalline samples. Residues of aliquots (1 ml.) of the standard and sample solutions of acetophenetidin and of caffeine were mixed, separately, with 400 mg. of 200/325 mesh and 20/40 mesh of potassium bromide for twenty and five minutes, respectively, using the vibrator-grinding procedure. Quantitative determinations were made using baseline absorbances of the bands at 8.52, 9.54, and 11.94 $\mu$  for acetophenetidin, and at 13.19 $\mu$  for caffeine.

## RESULTS AND DISCUSSION

In the authors' hands, the method of lyophilization proved unsuccessful as a means of dispersing samples in potassium bromide for quantitative studies. However, qualitative spectra of mixtures prepared in this way were essentially the same as those obtained using the two adopted procedures.

In preliminary experiments with potassium thiocyanate as an internal standard, disks of dispersions of this compound with ethinyl estradiol, hydrocorti-

sone, and reserpine and potassium bromide revealed the  $C \equiv N$  band at 4.87 $\mu$ . The spectra of these compounds differed from those in potassium bromide disks alone by the presence of this band. However, in disks of potassium thiocyanate with cortisone and potassium bromide, two bands of varying intensities appeared in the  $C \equiv N$  region at 4.81 and 4.87 $\mu$ . In addition, on heating disks of this mixture the 5.98 $\mu$  band attributed to the  $-C=C-C=O$  group of cortisone and the 4.87 $\mu$  band were seen to disappear. Extraction of these heated disks with chloroform and reformation of a potassium bromide disk from the extract residue revealed the normal spectrum of cortisone with the  $C=O$  band at 5.84 $\mu$  and the  $-C=C-C=O$  band at 5.98 $\mu$ . These observations are indicative of bonding or complex formation between the  $-C=C-C=O$  group of cortisone and the  $-C \equiv N$  group of potassium thiocyanate. Because of these variations, the difficulty of obtaining reproducible potassium bromide-potassium thiocyanate mixtures, and the ease of making accurate thickness measurements, the use of an internal standard was avoided.

Mixtures which were produced by hand-grinding crystalline samples and residues or by vibrator-grinding weighed residues with potassium bromide were not as quantitatively reproducible as, or required more time than, those prepared by the final methods.

In general, under the described experimental conditions, disks of hand-ground mixtures gave spectra with somewhat better resolution and more intense absorbance than disks of vibrator-ground mixtures. In Fig. 1 are seen qualitative spectra of the 2-15 $\mu$  region of some of the compounds studied. Although vibrator-grinding is mechanically more efficient, because of the grinding force exerted on the crystal, it is more likely to produce mixtures whose disks show spectral changes and distortions. Under conditions of greater than 50% relative humidity, the vibrator-grinding procedure suffers from inadequate mixing as a result of adsorption of moisture from the atmosphere. In addition, hand-grinding is preferable for those compounds which exhibit spectral changes on vibrator-grinding. When the hand-grinding procedure is accurately reproduced, the results are as reliable as those where vibrator-grinding is employed.

In general, the effects of particle size of the matrix on the ease of disk formation and on the qualitative spectrum agree with previous reports (1, 16). It was found that samples mixed with the coarse 20/40 mesh potassium bromide required less grinding, adsorbed less water, and produced clearer disks than did the 200/325 mesh powder. Vibrator-grinding of 200/325 or 20/40 mesh potassium bromide for longer than twenty minutes produced a powder of such small particle size that disk formation was difficult in that the disks were flaked and cracked when pressed from the die. With the vibrator-grinding procedure, those compounds which showed a tendency for spectral changes exhibited these changes with less grinding time with the 20/40 mesh crystals than when the 200/325 mesh powder was used. It is presumed that the larger particles of potassium bromide contribute to the grinding of the sample. As a result, anomalies may occur more readily than when the finer particles of potassium bromide are used.

<sup>1</sup>In recent experiments, the  $Ba(OH)_2$  layer has been omitted with an increase in the reproducibility of recoveries.

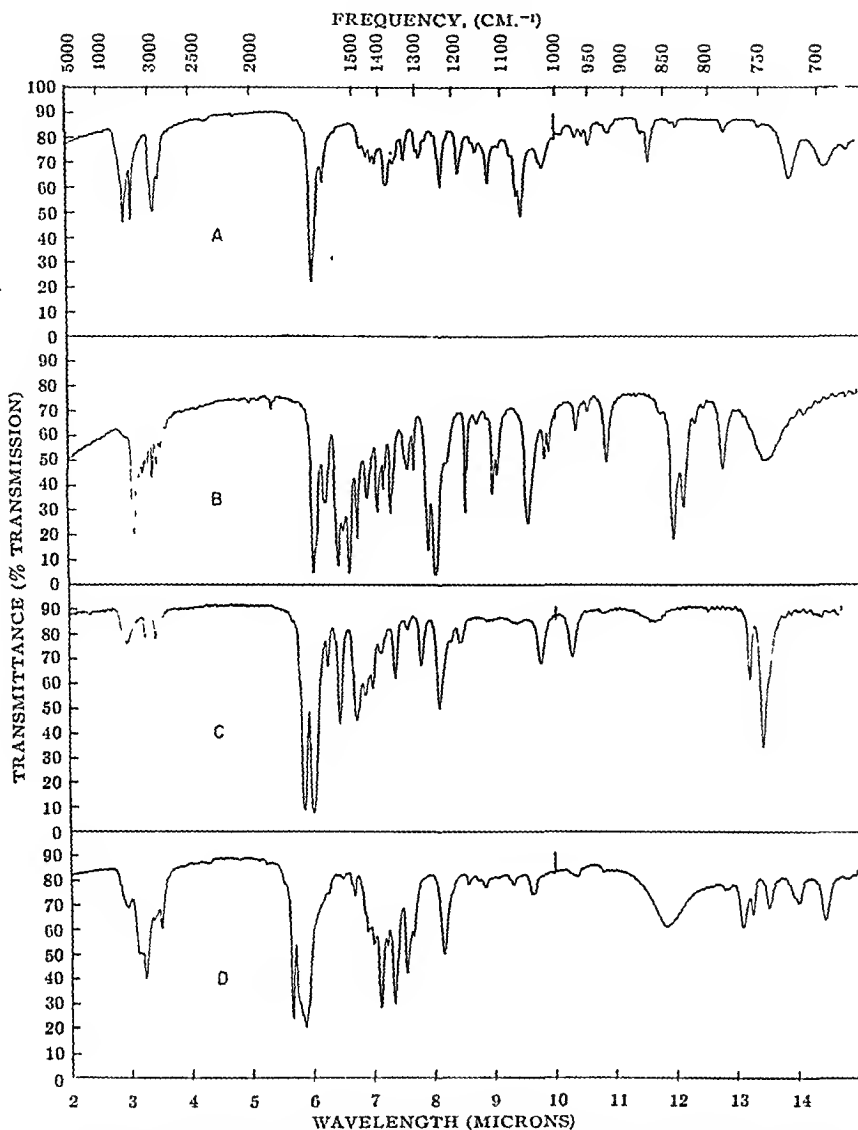


Fig. 1.—Infrared spectra of ethinyl testosterone from methanol (A), aceto-phenetidin from methanol (B), caffeine from benzene (C), and phenobarbital from benzene (D) in potassium bromide disks.

The clarity of the disks was a function of the amount of sample present, the size of the potassium bromide matrix, and the extent of grinding. Some compounds formed clear disks at concentrations up to 0.5 mg. per 200 mg. potassium bromide; disks of other compounds were cloudy at concentrations of about 0.2 mg. per 200 mg. potassium bromide. Cloudy disks could be used with satisfactory results as the baseline absorbance measurement eliminated the effect of background variation. Variable results were obtained when cracked or striated disks were compared with clear disks.

A potential source of absorbance measurement error is the presence of interference patterns in the spectrum. When these fringes were encountered, the baseline was drawn so as to bisect the highest

absorbing areas of the minima on each side of the band maximum.

A study of the changes in spectrum with grinding time and concentration reveals the stability of the compound under the grinding procedure. In Table I are given the absorptivity coefficients at different grinding times for some of the compounds studied. The optimum grinding time was chosen as the time producing maximum absorbance of the selected band and maximum spectral resolution with identity with the qualitative spectrum of the compound.

The authors' investigations indicated that the stability of a compound to the grinding conditions is a function of the crystal energy of the compound and of the stability of the disarranged crystalline lattice under the experimental conditions. Acetophe-

TABLE I—CHANGES IN ABSORPTIVITY COEFFICIENT WITH GRINDING TIME

Minutes	Absorptivity Coefficient			
	Ethinyl Testosterone, 6 03 $\mu$	Amobarbital, 8 07 $\mu$	Acetophenetidin, 11 94 $\mu$	Caffeine, 13 19 $\mu$
1				69
3	720	107		96
5	730		256	105
10	765	134	256	98
15	770	143	274	98
20		136	287	
25		137	272	

netidin (m p 135°) and amobarbital (m p 156–158 5°) did not exhibit spectral changes, however, phenobarbital (m p 174–178°), quimidine (m p 172–173°), and quinine (m p 174–175°) (10), showed variations in spectra with grinding time

The results of quantitative studies on standard ethinyl estradiol, hydrocortisone, 17-hydroxy-11-desoxycorticosterone, cortisone, reserpine, ethinyl testosterone, ascorbic acid, amobarbital, phenobarbital, acetophenetidin, and caffeine revealed the average deviation from linearity to be within  $\pm 4\%$  over the concentration range 0.05–0.40% by weight. The smallest variation of 1% was seen for ethinyl estradiol, hydrocortisone, cortisone, and ethinyl testosterone. The largest variation was seen with ascorbic acid and caffeine.

In Figs 2–4 are given the relationship between baseline absorbance and effective concentration for some of the compounds studied. In general, the absorbance, resolution, and the reproducibility of the spectra were improved by heating the disks. It is seen that the selected bands of acetophenetidin and caffeine follow the Beer-Lambert law within the described limits.

The 6.03 $\mu$  band of ethinyl testosterone shows linearity up to 0.065–0.10% by weight when the minima at 5.45 and 6.65 $\mu$  were used. Disks of higher concentrations vary from a straight line through the origin but show a straight line relationship with intercept at about 0.1 absorbance. In later work it was found that an improved relationship is obtained for the higher concentrations if band-broadening effects are considered. When the product of baseline absorbance times the apparent band width (in cm.) at one-half the height of absorbance is plotted against effective concentration (Fig 2), the 6.03 $\mu$  band follows the Beer-Lambert law within  $\pm 2\%$  for concentrations up to 0.25% by weight. Concentrations below or above 0.065% can be analyzed using baseline absorbance alone if the standard is of the same concentration. The 9.44 $\mu$  band showed deviations of  $\pm 4\%$  at concentrations up to 0.25% by weight.

For a given compound, all bands did not follow the Beer-Lambert law. In addition, for some compounds (caffeine and phenobarbital), bands which obeyed the Beer-Lambert law on direct determination gave nonlinear results when the standard compounds were carried through the separation procedures. Only those bands which showed linearity after the compound was subjected to the separation procedure were used.

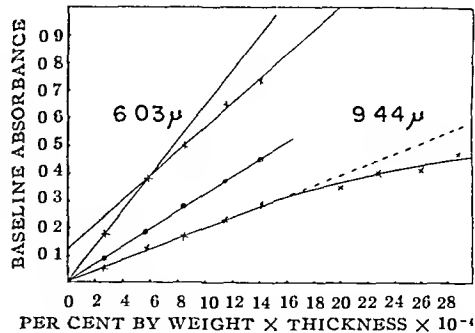


Fig 2—Calibration curves of ethinyl testosterone showing the relationship between baseline absorbance and effective concentration (X), and between baseline absorbance times apparent half-band width and effective concentration (O)

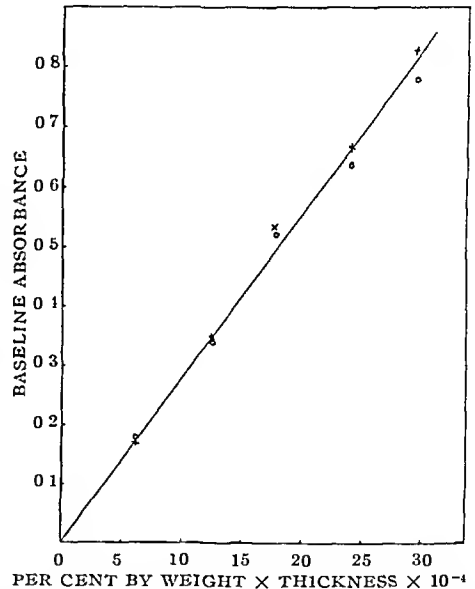


Fig 3—Calibration data for acetophenetidin (11.94 $\mu$ ) before (O) and after (X) heating potassium bromide disks.

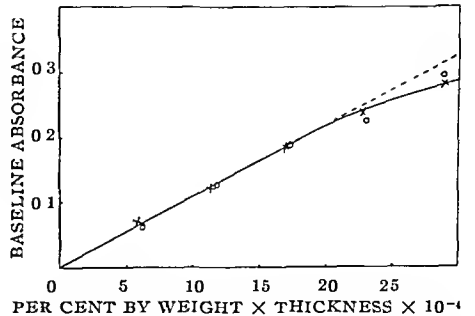


Fig 4—Calibration data for caffeine (13.19 $\mu$ ) before (O) and after (X) heating potassium bromide disks.

TABLE II.—K VALUES

Experiment No.	Ethinyl Testosterone, 6.03 $\mu$		Phenobarbital, 5.88 $\mu$	Acetophenetidin <sup>a</sup>		Caffeine <sup>a</sup>
	Hand-Ground/Vibrator-Ground		Hand-Ground	9.54 $\mu$	11.94 $\mu$	13.19 $\mu$
1	762, 762	664, 660	620	..	..	105
2	746, 760	640	614, 613	249, 256	261, 274	95, 105
3	758, 761	673	..	246	272	109.5
4	763, 761	..	..	..	..	..

<sup>a</sup> Vibrator-ground.

TABLE III.—TABLE OF RECOVERIES FOR ETHINYL TESTOSTERONE

Sample	Added or Declared mg./Tablet	Found per Tablet <sup>a</sup>					
		Ultraviolet		Hand-Ground		Infrared	
		mg.	%	mg.	%	Vibrator-Ground mg.	%
Standard	4.85	4.81	99.1	4.75	98.0	..	..
Standard	25	..	..	24.85	99.4	..	..
A	25	26.90	107.5	27.20	108.8	27.00	108.0
B	10	9.53	95.3	9.65	96.5	9.53	95.3
C	25	24.70	98.8	24.85	99.4	25.25	101.0
D	10	9.48	94.8	9.54	95.4	9.38	93.8

<sup>a</sup> Values represent the average of at least two determinations.TABLE IV.—ANALYSES OF SOME PHARMACEUTICAL COMPOUNDS AND PREPARATIONS<sup>a</sup>

Active Ingredient	Sample Description	Added mg. or Declared mg./Tablet	Found			
			Ultraviolet or Gravimetric, mg.	%	Infrared mg.	%
Hydrocortisone	Standard	2.02	..	..	2.00	99.0
Hydrocortisone + 17-hydroxy-11-desoxycorticosterone	Tablets	20.0	8.6 <sup>b</sup>	43.0	9.5	47.5
			11.2 <sup>b</sup>	56.0	10.5	52.5
Phenobarbital	Standard	50.0	48.1	96.2	47.3	94.6
Phenobarbital	Tablets	97.2	94.4	97.1	94.8	97.5
Phenobarbital + aminophylline	Tablets	32.4	31.6	97.5	32.1	99.0
Acetophenetidin	Standard	37.0	37.1	100.2	37.4	101.1
Acetophenetidin + acetanilid	Synthetic mix	1.0	..	..	0.994	99.4
		0.1	..	..	..	..
Acetophenetidin + acetanilid	Synthetic mix	1.0	..	..	0.990	99.0
		0.5	..	..	..	..
Acetophenetidin	Synthetic mix	37.0	38.6	104.3	38.5	104.1
Acetophenetidin	Tablets	162.2	146.5	90.3	147.0	90.6
Caffeine	Standard	7.34	7.27	99.0	6.9-7.56	94-103
Caffeine	Synthetic mix	7.36	7.84	106.5	7.75	105.2
Caffeine	Tablets	32.4	28.6	88.1	29.5	91.0

<sup>a</sup> These results are averages of two or more determinations. <sup>b</sup> Gravimetric.

In Table II are given some of the *K* values for the selected bands obtained from ethinyl testosterone, phenobarbital, acetophenetidin, and caffeine which were carried through the given procedures. It is seen that, except for caffeine, the values agree within 3% with the average values. A maximum of 8% variation from the average was shown by caffeine. The average *K* values of duplicate disks of the standard compounds were used in the determination of the corresponding pharmaceutical preparations.

The results which are given in Tables III and IV were corrected for the recoveries of the standards from the described procedures. In Table III are seen the results of ultraviolet and infrared determinations of ethinyl testosterone from recovery experiments and from analyses of tablets using the described procedure. On the basis of ultraviolet and infrared results, a 99% recovery was obtained of

this compound from the procedure. The analyses of several tablets reveal excellent agreement (within 1-2%) between ultraviolet and infrared data.

From Table IV, it is seen that there is a 3.5-4.5% variation between the infrared and gravimetric determinations of hydrocortisone and of 17-hydroxy-11-desoxycorticosterone. Acetophenetidin, as determined by infrared, agreed within 1% with those results determined by ultraviolet spectrophotometry. Analyses of quantitative mixtures of this compound with acetanilid showed 99% of the amount added. A 100% recovery was obtained when standard acetophenetidin was carried through the separation procedure.

The greatest disparity was seen between the ultraviolet and infrared determinations of caffeine. Although a 99% recovery of standard caffeine was obtained by ultraviolet determination, the recovery

as indicated by infrared determinations varied between 94 and 103%. As shown in Table IV, when an average *K* value is used, the infrared results from the analyses of a synthetic mixture agree within 1.2% with ultraviolet results. The analyses of a tablet mixture revealed an average variation of 3.0%. The fact that this compound became slightly discolored on evaporation to dryness after the separation may explain the larger variations.

Phenobarbital exhibited three spectra when attempts were made to apply the vibrator-grinding procedure. Because of this, the hand-grinding procedure was used in the analysis of this compound. Under the described procedure, a single reproducible spectrum was obtained. Comparison of the 5.88  $\mu$  carbonyl band of standard and sample carried through the same procedure gave results which agreed within 1% of the ultraviolet results. In the preparation containing aminophylline, there was a 1.5% difference between ultraviolet and infrared results. The recovery of standard phenobarbital from the column was 96% of the amount added.

Attempts to analyze reserpine and ascorbic acid in dosage forms were unsuccessful because of the instability of these compounds.

The effect of crystalline form on the infrared spectra of solids has been emphasized (1, 6, 20). Since the relationship between structure and vibration spectrum of a crystalline compound is based on the unit cell of the crystal and not on the individual molecules (13), it is apparent that the standard and sample must have the same crystalline form in order to produce identical infrared spectra. The methods described here insure the use of identical crystalline forms for standard and unknown by subjecting them to identical treatment. The inclusion of the steps for removing final traces of solvent and adsorbed water from the potassium bromide-compound mixture eliminates the effects of solvent bands on the spectrum.

The results show that the quantitative potassium bromide method can be used in the analysis of compounds of varied types. The agreement between the added amounts and the amounts detected when standards were subjected to the separation procedures is evidence of the excellent recovery for most of the compounds. The agreement between ultraviolet and infrared results on pharmaceutical preparations attests to the usefulness of this procedure. Provided anomalous spectra are not encountered, the results from analyses of potassium bromide disks can provide both quantitative determination and qualitative identification.

Because of the time involved in preliminary investigation, it is thought that this method will be most widely applicable and acceptable for the analyses of polar and isomeric compounds which cannot be

analyzed more easily by combining more conventional means of determination with identification by infrared spectroscopy.

## SUMMARY

These experiments show that the quantitative potassium bromide method can be applied in the analyses of compounds of varied origin. In general, the results agree within 1 to 4 per cent with the amount added or detected by other means in synthetic mixtures and in recovery experiments. Comparison of standard and sample subjected to the same separation procedure, the same solvent, and the same grinding regimen give infrared results which agree within 3 per cent with those obtained from more conventional methods of analysis of pharmaceutical preparations.

## REFERENCES

- (1) Baker, A. W., *J. Phys. Chem.*, **61**, 450 (1957).
- (2) Barker, S. A., Bourne, E. J., Weigel, H., and Whiffen, D. H., *Chem. & Ind. London*, **1956**, 318.
- (3) Barker, S. A., Bourne, E. J., Neely, W. B., and Whiffen, D. H., *ibid.*, **1954**, 1418.
- (4) Bent, H. A., and Crawford, B., Jr., *J. Am. Chem. Soc.*, **79**, 1793 (1957).
- (5) Browning, R. S., Wiberley, S. E., and Nachod, F. C., *Anal. Chem.*, **27**, 7 (1955).
- (6) Chapman, D., *J. Chem. Soc.*, **1957**, 2715.
- (7) Elsey, R. D., and Haszeldine, R. N., *Chem. & Ind. London*, **1954**, 1177.
- (8) Farmer, V. C., *ibid.*, **1955**, 586.
- (9) Hayden, A. L., *Anal. Chem.*, **27**, 1486 (1955).
- (10) Hayden, A. L., and Sammul, O. R., *THIS JOURNAL*, **49**, 497 (1960).
- (11) Ingebrigtsen, D. N., and Smith, A. L., *Anal. Chem.*, **26**, 1765 (1954).
- (12) Ito, A., and Amakasu, O., *J. Pharm. Soc. Japan*, **77**, 1083 (1957).
- (13) Jones, R. N., and Sandorfy, C., "Chemical Applications of Spectroscopy," Vol. 9, Interscience Publishers, Inc., New York, N. Y., 1956, p. 294.
- (14) Kirkland, J. J., *Anal. Chem.*, **27**, 1537 (1955).
- (15) Levine, J., *THIS JOURNAL*, **46**, 687 (1957).
- (16) Milkey, R. G., *Anal. Chem.*, **30**, 1931 (1958).
- (17) Nicholson, D. E., *ibid.*, **31**, 519 (1959).
- (18) Padgett, W. M., II, Talbert, J. M., and Hamner, W. F., *J. Chem. Phys.*, **26**, 959 (1957).
- (19) Pliskin, W. A., and Eischens, R. P., *J. Phys. Chem.*, **59**, 1156 (1955).
- (20) Roberts, G., *Anal. Chem.*, **29**, 911 (1957).
- (21) Rosenkrantz, H., Potvin, P., and Skogstrom, P., *ibid.*, **30**, 975 (1958).
- (22) Sabatino, F. J., *J. Assoc. Offic. Agr. Chemists*, **37**, 1001 (1954).
- (23) Schiedt, U., and Reinwein, H., *Z. Naturforsch.*, **7b**, 270 (1952).
- (24) Schiedt, U., *ibid.*, **8b**, 66 (1953).
- (25) Schwarz, H. P., Childs, R., Dreisbach, L., and Mastangelo, S. V., *Science*, **123**, 328 (1956).
- (26) Stinson, M. M., and O'Donnell, M. J., *J. Am. Chem. Soc.*, **74**, 1806 (1952).
- (27) Susi, H., and Rector, H. E., *Anal. Chem.*, **30**, 1933 (1958).
- (28) White, J. W., Jr., Eddy, C. R., Petty, J., and Hoban, N., *ibid.*, **30**, 506 (1958).
- (29) Wiberley, S. E., Sprague, J. W., and Campbell, J. E., *ibid.*, **29**, 210 (1957).

# Infrared Analysis of Pharmaceuticals II\*

## A Study of the Cinchona Alkaloids in Potassium Bromide Disks

By ALMA L. HAYDEN and OSCAR R. SAMMUL

In the course of an investigation into the applicability of a quantitative potassium bromide disk method to various drugs and chemicals, the cinchona alkaloids, quinidine, quinine, cinchonine, and cinchonidine were studied. Under certain experimental conditions, possibly due to the formation of dimorphous and amorphous forms, anomalous spectra were obtained for quinidine and quinine. Cinchonine and cinchonidine did not exhibit significant variations in spectra under the conditions investigated.

BECAUSE of the similarity in structure between quinidine and quinine, and between cinchonine and cinchonidine, and because of rotational isomerism, the infrared spectra of the two isomers of each series in carbon disulfide or chloroform solutions (Figs. 1 and 2) are very similar. As a result, these spectra are not easily differentiated, nor do they afford unequivocal quantitative determination when the presence of the isomer is possible.

The publication in 1952 (13, 14) of the potassium bromide disk method has resulted in successful applications to compounds of varied origin. However, there have been several reports (1-3, 6, 10-12) of anomalous spectra obtained with this method. In general, the origins of these anomalies may be placed in four groups: (a) formation of polymorphic crystalline forms, (b) formation of amorphous materials, (c) formation of hydrates, and (d) chemical transformations or combinations.

When an attempt was made to apply a quantitative potassium bromide method (7) to four cinchona alkaloids, anomalies of groups (a) and (b) were indicated with quinidine and quinine. Cinchonine and cinchonidine were stable under the conditions studied.

### EXPERIMENTAL

Quinidine was purified by formation of the tartrate and the hydrioidide, followed by neutralization, and recrystallization from benzene. Quinine was obtained by formation of the oxalate from the recrystallized hydrochloride, with subsequent neutraliza-

tion with ammonium hydroxide, and recrystallization from benzene. Cinchonine was purified by the mercuric acetate process (15), and was recrystallized from boiling alcohol or from benzene. Cinchonidine was purified by formation of the tartrate and subsequent neutralization. Recrystallization was effected from one part alkaloid in thirty parts benzene or in six parts alcohol. The recrystallized alkaloids were dried at 105° under high vacuum. Their melting points (uncorrected) on a Fisher-Johns melting block were: quinidine, 172-173°; quinine, 174-175.5°; cinchonidine, 206-207°; and cinchonine sublimed with decomposition, about 245°. In a sealed tube, cinchonine melts at 255°.

The spectra were obtained with a Perkin-Elmer model 21 double-beam spectrophotometer with sodium chloride optics. The spectra of the carbon disulfide solutions (less than 3 mg. per ml.) were determined using a 3-mm. microcell compensated with a variable space cell. The spectra of the chloroform solutions (3-10 mg. per ml.) were determined with 1-mm. macrocells.

In general, the potassium bromide mixtures and disks for qualitative and quantitative spectra were prepared as described (7) from aliquots of a benzene or methyl alcohol solution (1.0 mg. per ml.). For quantitative determinations, hand-grinding of solutions for ten minutes or vibrator-grinding of residues, which had been heated at 105°, for five minutes was employed. The resulting potassium bromide mixtures and disks were heated at 105° and the spectra were measured.

### RESULTS AND DISCUSSION

When the four recrystallized alkaloids were dispersed in potassium bromide under moderate conditions (7), their infrared spectra (Fig. 3) were distinctly different. Disks of cinchonine and cinchonidine (Fig. 4) followed the Beer-Lambert law within  $\pm 3.0\%$  up to a concentration of 0.4% by weight at 13.08 and 13.19 $\mu$ , respectively.

Quinidine, recrystallized from benzene and dried at 105° in vacuum, gave spectrum A of Fig. 5 when 1 mg. was ground for one or two minutes with 200 mg. of potassium bromide. Spectrum B was obtained on vibrator- or hand-grinding this mixture for five or ten minutes, respectively. In addition spectrum B was obtained when quinidine was vibrator-ground, and the fused product was dispersed in potassium bromide under moderate conditions. On microscopic examination, this fused product appeared to be a mixture of crystals and amorphous material.

Continued grinding of form B resulted in partial reversion to form A. The rates of the transformations varied with the amounts of starting materials, the grinding time, and the force applied by hand or by the steel balls in the vibrator. In Fig. 6 are

\* Received October 30, 1959, from the Division of Pharmaceutical Chemistry, Food and Drug Administration, Washington, D. C.

The authors are greatly indebted to Jonas Carol for his advice and criticism throughout this investigation.

The crystallographic determinations by Arnold Schulze are gratefully acknowledged.

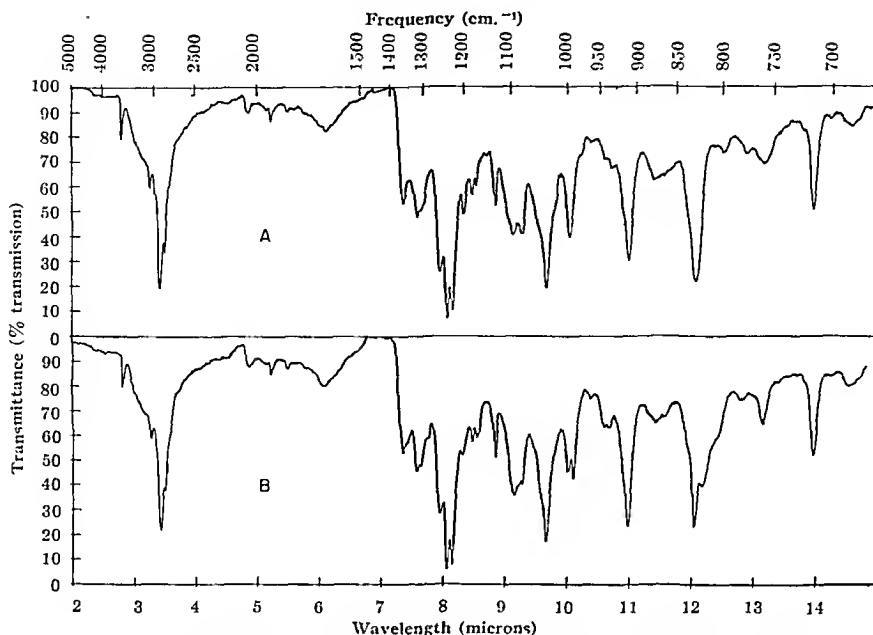


Fig. 1.—Infrared spectra of quinidine (*A*) and quinine (*B*) in carbon disulfide.

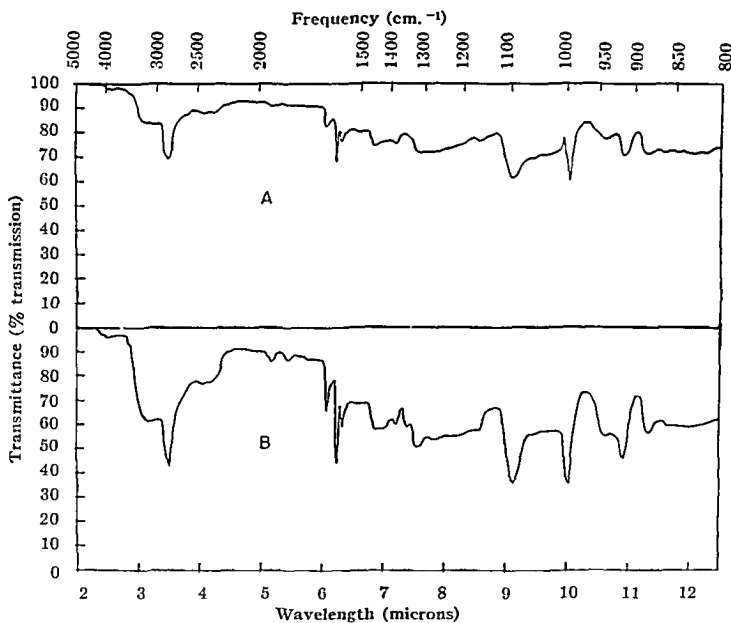


Fig. 2.—Infrared spectra of cinchonine (*A*) and cinchonidine (*B*) in chloroform.

shown some changes in spectrum in the 6–9 $\mu$  region with grinding time.

There have been reports of differences in the spectra of polymorphic forms of various compounds (4, 5, 9). Chapman (4) obtained the lowest melting forms of certain glycerides by quenching the liquids to 0°. Higher melting forms were detected by heating the quenched material or by cooling the liquid, while rapidly scanning parts of the infrared spectrum.

When quinidine was melted under anhydrous

conditions under vacuum and the melt was quenched at 0° to –2°, the resulting glassy material was faintly yellow and gave spectrum *B* of Fig. 5. On a Fisher-Johns melting block, under 10 $\times$  magnification, the quenched quinidine showed a transition point with partial melting at 74–76°, resolidification and gradual opaqueness above 85°, and final melting at 171–172°. The transition at 74–76° was not as apparent for the ground quinidine. However, spectrum *A* of Fig. 5 was obtained when this material was heated

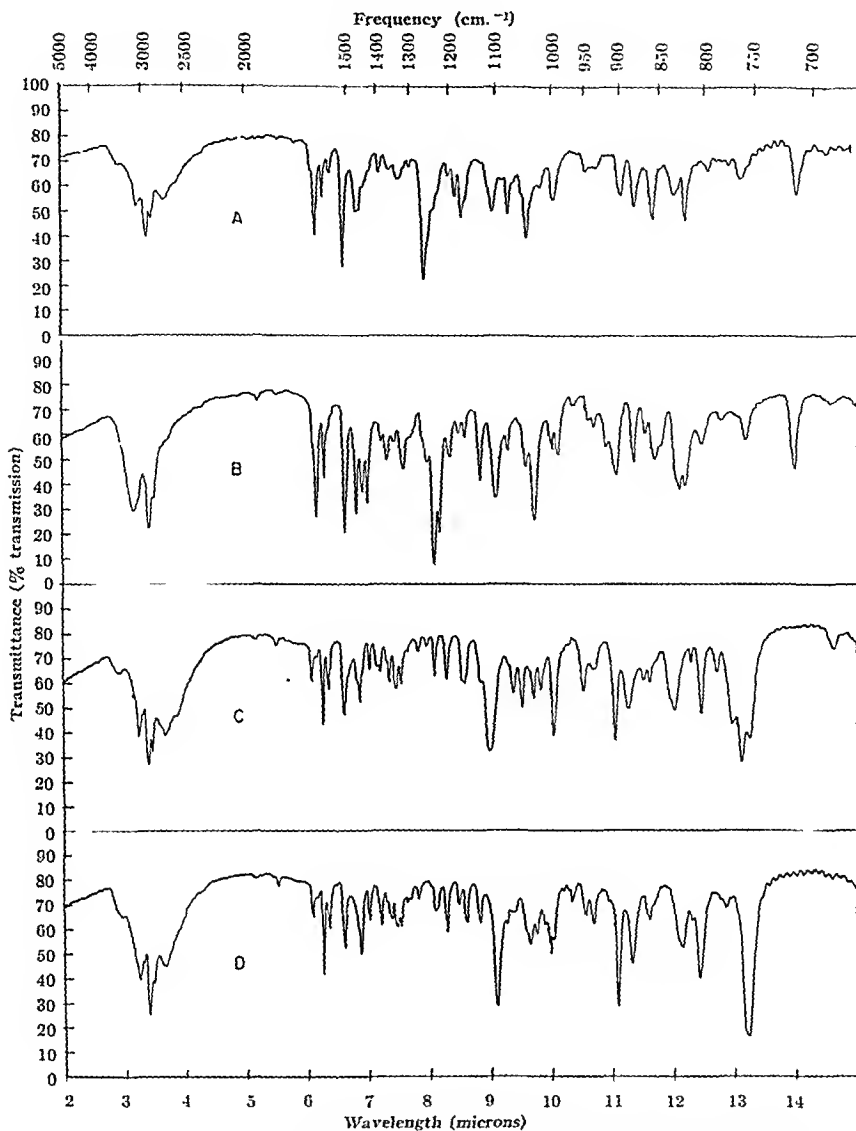


Fig. 3.—Infrared spectra of quinidine (A), quinine (B), cinchonine (C), and cinchonidine (D) in potassium bromide disks.

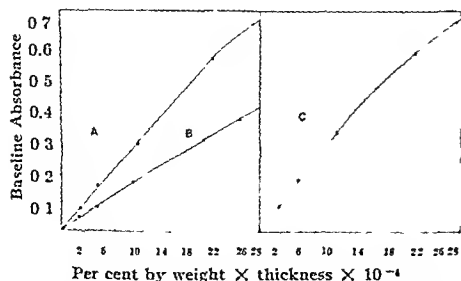


Fig. 4.—Calibration curves of cinchonidine at  $13.19\mu$  (A), cinchonine at  $13.08\mu$  (B), and quinine at  $8.02\mu$  (C) in potassium bromide disks.

at that temperature for thirty minutes. The ground quinidine became opaque above  $85^\circ$  and

melted at  $170$ – $171^\circ$ . Spectrum A was obtained from both final melting materials and from disks of form B which were heated at  $75^\circ$  or above.

Presumably, form B is produced as a result of localized heating and cooling during the grinding process. As suggested by Roberts (12), it is possible that as the sample is subjected to frictional forces during grinding, the local temperature is raised above the melting point of the compound. As these forces are removed from the crystals, immediate cooling might effect a rearranged form which is stable up to  $75^\circ$ . At higher temperatures, rearrangement to the crystal lattice of the higher-melting form could occur. This view is supported by the experiments with the quenched melt, by the fused condition of quinidine when ground under extreme conditions, and by the transition at  $74$ – $76^\circ$ .

The spectrum of form B is somewhat similar to



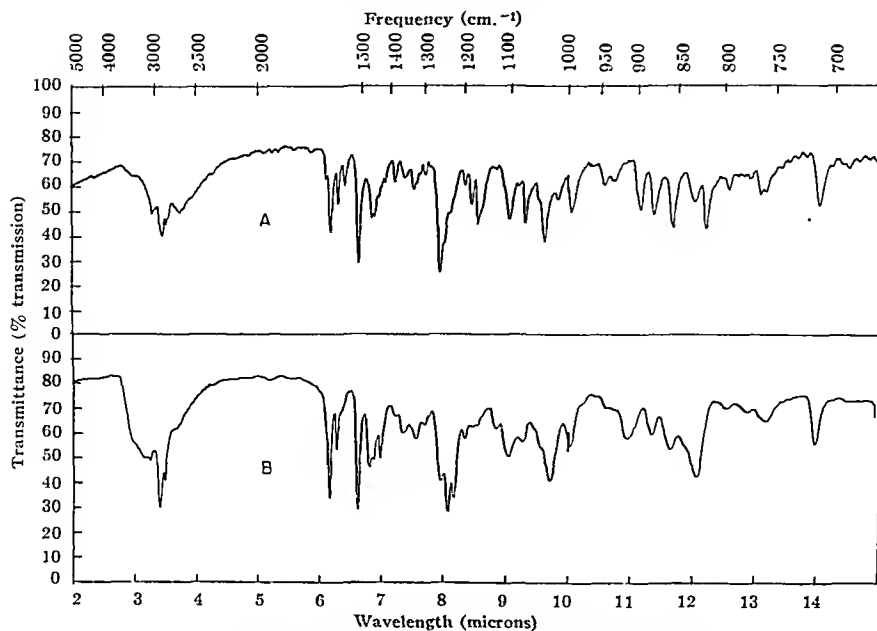


Fig. 5—Infrared spectra of quinidine hand-ground (*A*), and vibrator-ground or quenched melt (*B*) in potassium bromide disks.

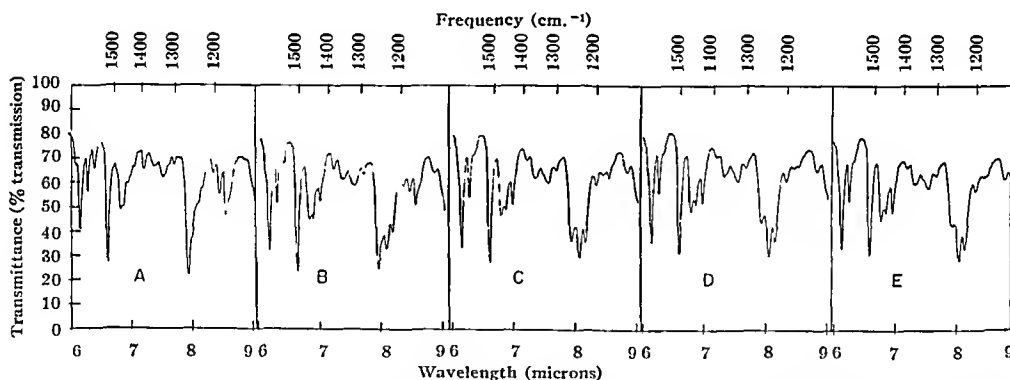


Fig. 6.—The 6-9 $\mu$  spectral region of quinidine in potassium bromide disks after grinding for one minute (*A*), fifteen minutes (*B*), thirty minutes (*C*), forty-five minutes (*D*), and after melting and quenching (*E*).

those of quinidine recrystallized from 95% ethyl alcohol and from water (Fig. 7). Therefore, the similarity of its spectrum to those of disks of quinine and of quinidine and quinine in solution is not construed as the result of amorphous material alone in the ground or quenched quinidine. When the crystalline solvates were dried at 105° in vacuum, there was a loss in weight equivalent to 1 mole ethyl alcohol and 1½ moles of water, respectively, and reversion to form *A*. Crystallographic examinations of these compounds were incomplete; only the  $\alpha$  and  $\gamma$  refractive indexes were determined. Although the  $\alpha$  or  $\gamma$  refractive index of the solvates and those of form *A* were different, the indexes of form *B* were similar to those of form *A*.

Hydrate formation, as an explanation of spectrum *B* of quinidine, was ruled out when the ground or quenched material was dried at 105° under vacuum for three hours with reversion to form

*A* without loss in weight. Compound decomposition or structural rearrangement is unlikely because of the ease of conversion from one form to the other. In addition, the appearance of form *B* on melting and quenching, the final melting point of the reverted quinidine, and the optical rotation values shown in Table I, make the possibility of significant decomposition or rearrangement remote.

TABLE I.—OPTICAL ROTATION VALUES

Sample	( $\alpha$ ) <sub>D</sub> in Absolute Ethanol, 24°C.
Quinidine	+258°
Quinidine grind	+253°
Quinidine melt	+257°
Quinidine melt heated	+254°
Quinidine. EtOH	+253°
Quinidine. 1½ H <sub>2</sub> O	+259°

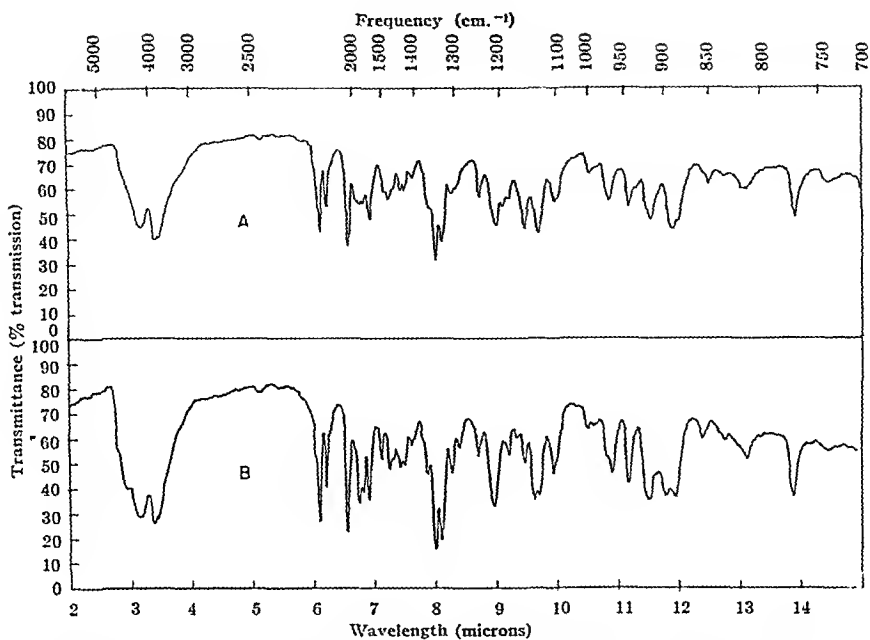


Fig. 7.—Infrared spectra of quinidine recrystallized from 95% ethyl alcohol (*A*), and from water (*B*) in potassium bromide disks.

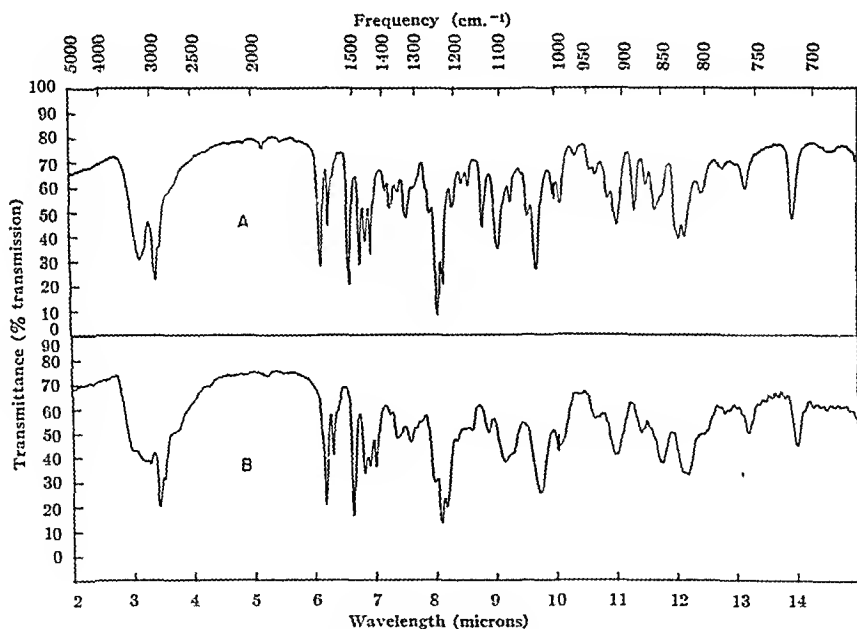


Fig. 8.—Infrared spectra of quinine moderately ground (*A*), and vigorously ground or melted and quenched (*B*) in potassium bromide disks.

Although the presence of dimorphous material in form *B* is indicated, an unequivocal assignment must await additional information, including complete crystallographic data. However, as the relationship between the structure and vibration spectrum of a crystalline compound is based on the unit cell of the crystal (8), the observed differences between the two spectra of quinidine are understandable.

Somewhat less spectral variations were encountered with disks of quinine (Fig. 8). The grind and the quenched melt were obtained under conditions similar to those described for quinidine. However, the transformations were controlled by hand-grinding and by heating disks of quinine at temperatures above 75°. The relationship between absorbance at 8.02 $\mu$  and effective concentration is shown in

It has been suggested (1) that the occurrence of polymorphism with the potassium bromide method is related to the crystal energy of the compound and to the stability of the polymorphic form. Significantly, the spectrum of the quenched melt of cinchonine is identical with that of the recrystallized alkaloid. Except for increased absorbance at  $10.92\mu$  at the expense of the  $11.05\mu$  band, the spectra of the quenched melt of cinchonidine and the starting material were the same. There were no significant changes in the spectra of these two alkaloids after vibrator-grinding for periods up to forty-five minutes. The stability of acetophenetidin (m.p.  $135^\circ$ ) and amobarbital (m.p.  $158^\circ$ ) (7), under the given experimental conditions, reveals that spectral variations are not dependent on melting point.

Some attempts to control the conversion of quinidine by reducing the grinding time and force, and by vibrator-grinding at  $80^\circ$  were not quantitatively reproducible. Work is in progress on a method of determination by ultraviolet or infrared spectrophotometry and identification by infrared spectroscopy of potassium bromide disks.

### SUMMARY

Dimorphous and amorphous forms of quinidine and quinine are indicated when these compounds are ground with or without potassium bromide. By heating quinine-potassium bromide mixtures and the resulting disks, a linear relation-

ship between absorbance and concentration times thickness is obtained. Quantitative determinations of quinidine in potassium bromide are variable.

Cinchonine and cinchonidine do not exhibit significant spectral variations under the experimental conditions. Their disks follow the Beer-Lambert law at concentrations up to 0.4 per cent by weight.

### REFERENCES

- (1) Baker, A. W., *J. Phys. Chem.*, **61**, 480 (1957).
- (2) Barker, S. A., Bourne, E. J., Weigel, H., and Whiffen, D. H., *Chem. & Ind. London*, **1956**, 318.
- (3) Barker, S. A., Bourne, E. J., Neely, W. B., and Whiffen, D. H., *ibid.*, **1954**, 1418.
- (4) Chapman, D., *J. Chem. Soc.*, **1957**, 271S.
- (5) Ebert, A. A., and Gottlieb, H. B., *J. Am. Chem. Soc.*, **74**, 2806 (1952).
- (6) Farmer, V. C., *Chem. & Ind. London*, **1955**, S86.
- (7) Hayden, A. L., and Sammul, O. R., *THIS JOURNAL*, **49**, 489 (1960).
- (8) Jones, R. N., and Sandorfy, C., "Chemical Applications of Spectroscopy," Vol. 9, Interscience Publishers, Inc., New York, N. Y., **1956**, p. 294.
- (9) Kendall, D. N., *Anal. Chem.*, **25**, 382 (1953).
- (10) Padgett, W. M., II, Talbert, J. M., and Hamner, W. F., *J. Chem. Physics*, **26**, 959 (1957).
- (11) Pliskin, W. A., and Eischens, R. P., *J. Phys. Chem.*, **59**, 1156 (1955).
- (12) Roberts, G., *Anal. Chem.*, **29**, 911 (1957).
- (13) Schiedt, U., and Reinwein, H., *Z. Naturforsch.*, **7b**, 270 (1952).
- (14) Stimson, M. M., and O'Donnell, M. J., *J. Am. Chem. Soc.*, **74**, 1805 (1952).
- (15) Thron, H., and Dirscherl, W., *Ann. Chem. Liebigs*, **515**, 252 (1935).

## Preparation of Tritium-Labeled Halothane (2-Bromo-2-chloro-1,1,1-trifluoroethane)\*

By CALVIN HANNA

Halothane was labeled with tritium (Wilzbach method) by exposure to 2.3 curies of tritium gas for fourteen days. The one hydrogen of the molecule was labeled and on purification gave a final specific activity of 2.9 mc. per Gm. In the preparation a considerable amount of 'loose' tritium and highly active impurities were separated and the purity of the final product following gas chromatographic purification was better than 99.95 per cent.

**HALOTHANE**<sup>1</sup> (CF<sub>3</sub>-CHBrCl) is receiving attention as a new general anesthetic agent (1). This compound gives fast inductions and

recoveries and has been used safely in over five thousand patients (2). Due to the anesthetic potency of this material the inhaled concentration should not exceed two per cent (2) which means rather low blood levels for an anesthetic agent. Due to the chemical stability of halothane the chemical procedure for its determination in blood and tissues is difficult and time consuming (3). An alternate approach to this problem is to use isotopically-labeled halothane as a tracer in designing and testing new methods for its determination. Tritium-labeled halothane was prepared by the Wilzbach method (4) and this report covers the purification and determination of the chemical purity of the final labeled product.

\* Received November 27, 1959, from the University of Vermont, College of Medicine, Burlington.

This investigation was supported by a senior research fellowship, SF-156, from the Public Health Service.

<sup>1</sup> Fluothane is the registered trade name of halothane of Ayerst Laboratories, Inc., New York, N. Y.

## PROCEDURES

**Tritiation.**—Two and one-half milliliters of halothane (Fluothane, Ayerst Laboratories, Inc., New York, N. Y., containing 0.01% thymol and less than 0.005% impurities) was sealed in a 20-ml. round-bottom flask (fitted with a break seal), with 2.3 curies of tritium gas at 27° and 0.39 atm for fourteen days. The flask was cooled in liquid nitrogen, opened and the excess tritium gas pumped off. The remaining radioactivity was 85 mc of tritium per ml. of halothane.

**Purification.**—The flask was rescaled and a ground-glass joint fused to the open end of the break seal. A long condenser was attached and 20 ml of ice cold water was poured into the condenser. Halothane is very volatile and this heavy liquid was kept under water in the cold. After the flask was cooled the break seal was smashed with a long glass rod through the condenser. The contents of the flask were shaken for one hour and a sample of the organic layer was assayed, giving an activity of 18 mc. per ml, with the remaining activity being in the aqueous layer.

For purification a 1-ml aliquot of the organic layer was mixed with 9 ml of nonlabeled halothane under 10 ml of cold water. After extracting the organic layer with four additional 10-ml aliquots of water in order to remove any remaining loose tritium, the organic layer was again assayed, giving a specific activity of the diluted material of 1.4 mc. per ml. Next the organic layer was washed with 10-ml. aliquots of dilute hydrochloric acid, dilute sodium hydroxide, and finally with water. The organic layer was then distilled from 20 ml of water, giving an activity of 1.0 mc per ml. A sample of this was subjected to gas chromatographic separation and analysis. The organic layer was fractionated by one pass through a dinonyl phthalate column (stage 1) and then by two passes (stages 2 and 3) through a polyethylene glycol column. The results were as follows:

(a) Stage 1, 'light' fraction (at 2% of the total volume) containing halothane with normal halothane impurities

(b) Stage 1, 'heavy' fraction (at 1% of the total volume) containing halothane with any impurities of longer retention time. The main fraction in stage 1 was refractionated

(c) Stage 2, 'heavy' fraction (at 10% of the total volume) containing water, impurities, and halothane. The main fraction from stage 2 was refractionated.

(d) Stage 3, 'heavy' fraction (at 2% of the total volume) containing traces of halothane, impurities, and water.

(e) Stage 3, 'main' fraction (at 85% of the total volume) containing chromatographically pure (less than 0.005% impurity) halothane

Of the total radioactivity in the fractionated sample (a) had 29%, (b) 14%, (c) 2%, (d) trace, and (e) the main fraction 55%. The specific activity of the original halothane before dilution was 2.9 mc/Gm. The purity of the diluted (1:10 v/v) halothane was better than 99.995%, or the purity of the original product was better than 99.95%.

**Counting.**—Halothane-tritium was counted in a liquid scintillation counter (Packard Tri-Carb) using toluene-tritium as the internal standard. The counting solution was composed of 4 Gm./L. of DPO (2,5-diphenylazole) and 100 mg./L. of POPOP [1,4-di(5-phenyloxazolyl)benzene] in toluene

## DISCUSSION

Some of the original activity could have been due in part to the labeling of the thymol 0.01% and traces of normal halothane impurities of less than 0.005%. The thymol should have been removed during the repeated washings and on distillation. Some halothane was lost with the repeated washings since the solubility at 20° in water is 3.45 mg./ml. To reduce this loss the extractions were carried out in the cold at near 0° and in the final step excess water was separated mechanically by freezing in a dry ice-acetone mixture with the organic layer being decanted from the ice crystals. The vapor pressure of this liquid presents problems in purification since it is quite volatile and must be transferred to pre-cooled equipment. The vapor pressure follows the equation  $\log p = 7.689 - (1555/T)$ , where  $p$  is in mm Hg and  $T$  is in absolute temperature units.

The diluted halothane-tritium can be counted in a liquid scintillation counter at 22% efficiency and it can be assayed in concentrations of less than 0.1 mg with suitable statistical accuracy. This material is being used to calibrate an infrared method of measuring halothane after it is extracted from blood.

## REFERENCES

- (1) Raventos, J., *Brit. J. Pharmacol.*, **11**, 394 (1956)
- (2) Abujian, J., Jr., Brazell, E. H., Dente, G. A., and Mills, E. L., *J. Am. Med. Assoc.*, **171**, 535 (1959)
- (3) Duncan, W. A. M., *Brit. J. Anaesthesia*, **31**, 316 (1959)
- (4) Witzbach, K. E., *J. Am. Chem. Soc.*, **79**, 1013 (1957).

# A Study of Aqueous Medicinal Lubricants\*

By GERHARD LEVY† and T. W. SCHWARZ

The relation of viscosity and solid concentration to the lubricant effect of aqueous solutions of methylcellulose was investigated. Lubricity increased directly with solid concentration, while the high viscosity at greater methylcellulose concentrations prevented any further lubricity increase. Glycerin, polyethylene glycol 400, and propylene glycol are effective lubricants over a wide concentration range under the conditions tested.

THE FORMULATION of aqueous jellies and liquids designed to reduce the friction as well as the resulting pain and trauma encountered when inserting various devices into anatomical passages involves a consideration of the qualitative and quantitative effect of potential constituents on the lubricating property of the final product. In addition, the relationship between the rheologic characteristics of the product and its lubricating property must be established.

We have recently described a method for the measurement of the lubricating property of medicinal products by means of a specially designed instrument, the lubrimeter (1). The present report deals with a study of aqueous medicinal lubricants and the effect of viscosity, concentration of hydrocolloid, and addition of a polyol on lubricating property.

Friction, the resistance to motion when one solid body slides over another, is due to the junctions formed at the regions of real contact between the two bodies (2). Such areas of contact are very small relative to the actual surface area and involve only the tips of the asperities that are found on even the "smoothest" of surfaces. Ordinarily, the real area of contact is determined primarily by the load and the yield pressure of the materials and is not dependent on surface area. In the case of elastic or viscoelastic materials such as polyethylene or epithelium, the area of contact is no longer directly proportional to the load and as a result, the coefficient of friction increases as the load is reduced (2). For this reason, it is important that medicinal lubricants be tested under much lower loads than those utilized in evaluating engine lubricants.

A basic law of friction states that it is proportional to the load between surfaces. This law,

however, holds only for dry friction, where the opposing surfaces are free of any film (3). It does not directly apply to skin and mucous membranes, another reason why the load must be kept low enough to be within the range commonly encountered in clinical practice.

For the purpose of this investigation of the relationship between viscosity and lubricant effect a series of solutions of two grades of methylcellulose, one a high molecular weight polymer and the other a low molecular weight polymer, were prepared and their lubricant effect evaluated. The two series of solutions may be expected to exhibit one basic type of flow characteristics. The individual solutions differ primarily in their viscosity and solid content. It was also our purpose to determine the lubricating effect of polyols, such as propylene glycol and glycerin, since these substances are common constituents of lubricating jellies, liquids, and lotions. The lubricating effects of the pure polyols, of their aqueous solutions, and of methylcellulose solutions containing a given concentration of a polyol were established.

## EXPERIMENTAL

**Apparatus.**—The "Lubrimeter" consists of a polished brass roller which is rotated by a synchro-motor at various selected constant speeds. On this roller rests a drum which is covered with ribbed polyethylene sheeting. The drum is free to rotate around its own axis. The roller is partly immersed in a pan that serves as a reservoir for the lubricant. As the roller rotates, the drum turns in the opposite direction as a result of friction. The torque exerted on the drum is a measure of the frictional force and is determined by means of selected springs calibrated in grams. A detailed description of the instrument can be found in our previous report (1).

**Procedure.**—The procedure is identical to the one previously described (1). The reported friction values were determined at  $25^\circ \pm 2.5^\circ$  and were average values obtained from two series of six determinations each. The difference between the average values of the two series was less than 3%.

**Materials.**—The solutions were prepared from Methocel 90 HG 4000 and Methocel 90 HG 400 (hydroxypropyl methylcellulose)<sup>1</sup> by adding the proper amount to distilled water previously heated to about  $80^\circ$ . The water contained 0.15% methylparaben and 0.025% propylparaben as preservatives. The solutions were then placed in a refrigerator for twenty-four hours.

Glycerin, propylene glycol, and polyethylene glycol 400 were of U. S. P. grade.

<sup>1</sup> Dow Chemical Co., Midland, Mich.

\* Received August 21, 1959, from the University of California, School of Pharmacy, San Francisco 22.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

† Present address: University of Buffalo, School of Pharmacy, Buffalo 14, N. Y.

**Viscosity Determinations.**—Viscosity measurements were made with the Brookfield viscometer, model LVF, at 25°, 60 r. p. m., after ten minutes of spindle rotation. The Brookfield viscosity units shown in Fig. 1 would, in the case of Newtonian liquids, represent centipoise.

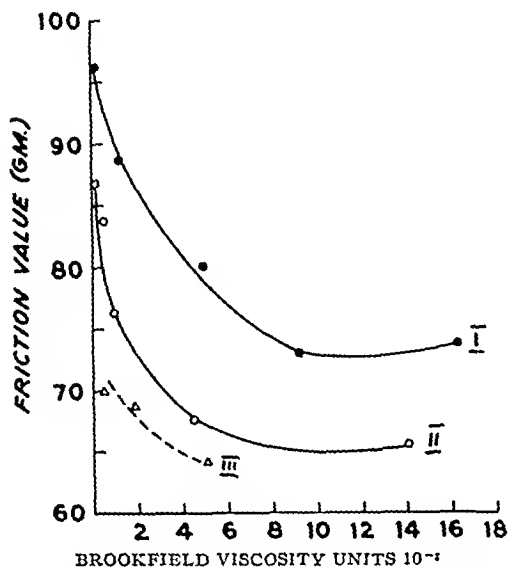


Fig. 1.—The relation of viscosity to lubricity. ●—● Methocel 90 HG 4000; ○—○ Methocel 90 HG 400; Δ—Δ—Δ Methocel 90 HG 4000 with propylene glycol 20% v/v.

## RESULTS AND DISCUSSION

The relationship between the viscosity and lubricant effect of methylcellulose solutions is shown in Fig. 1. Curve I represents solutions of Methocel 90 HG 4000, while curve II represents solutions of the lower molecular weight polymer, Methocel 90 HG 400. The rapid initial increase in lubricity, indicated in Fig. 1 by a decline of friction value, levels off at higher viscosities. In fact, curve I indicates a range of maximum lubricant effect and a slight decrease in lubricity at viscosities above the optimum range. It must be recognized that quantitatively this optimum range holds true only under the conditions of the test, and may vary with load and velocity of the moving surface.

The significance of viscosity in lubrication has been studied extensively in the engineering sciences. In instances of hydrodynamic ("full fluid") lubrication, the moving surfaces are completely separated by a continuous film of lubricant. Since it is generally accepted that there is no slip of the liquid at the solid surfaces, the resistance to motion (the resisting torque in the lubricimeter) is proportional to the coefficient of viscosity (4). If the lubricant exhibits Newtonian flow, the resistance to motion is also proportional to the velocity at which one solid surface moves relative to the opposing surface. These relationships are essentially based on Newton's law of viscous flow. Thus, resistance to motion is also inversely proportional to the clearance

between opposing surfaces. These factors along with dimensional considerations constitute Petroff's equation which deals with the resisting torque encountered in lubricated bearings (5). This equation has been shown to provide reasonably accurate estimates of actual friction.

On the basis of the foregoing discussion, it appears that the lower the viscosity of the lubricant, the lower the resistance to motion. This is true only up to a certain limit. As the viscosity of the lubricant decreases, its "load-carrying" capacity also diminishes, and the distance between the opposing surfaces is reduced. If this distance becomes less than the height of the surface irregularities, direct contact between the opposing surfaces may be established and the coefficient of friction becomes much larger. The low "load-carrying" capacity at low viscosities and the resistance to motion encountered at high viscosities account for a range of optimum viscosity for lubrication under specified conditions.

The correlation of lubricity with the viscosity of non-Newtonian liquids, such as solutions of methylcellulose, is much more complex than the straightforward relation of viscosity of Newtonian liquids to their lubricant effect. The rate of shear, to which the solutions are subjected when they exist as a lubricant film between opposing surfaces, will vary extensively throughout the film because the clearance between the opposing surfaces varies widely and changes constantly. The viscosity of methylcellulose solutions changes accordingly, since the apparent viscosity coefficient of non-Newtonians is dependent on the rate of shear. Our choice of shear rate for the determination of viscosity had to be arbitrary and, therefore, the correlation of non-Newtonian viscosity with lubricant effect can, at best, be only descriptive.

A comparison of curves I and II in Fig. 1 shows that, at equal viscosities, the lubricant effect of the two grades of methylcellulose differs markedly. The solid content of the Methocel 400 solutions is much greater than that of the Methocel 4000 solutions of equal viscosity by reason of the lower degree of polymerization of the former. Figure 2 shows a plot of friction values against per cent solid content. Significantly, the lubricant effect increased (i. e., friction values decreased) in linear fashion with increasing solid content. Moreover, there was relatively little difference between the two grades of methylcellulose; they could almost be plotted on the same curve. This linear relationship holds true up to a certain point, at which a sudden cut-off or levelling-off occurs. By comparison with Fig. 1, it can be seen that this cut-off occurs when the viscosity becomes sufficiently high to interfere with lubrication.

The data appear to indicate that the lubricant quality of methylcellulose solutions is primarily a function of their solid content. High viscosity as a result of increasing solid concentrations limits further lowering of the friction value. Beyond a certain viscosity, lubricity does not increase and apparently even decreases, as curve I, Fig. 1 indicates.

These observations may be explained in the light of the experiences gained by lubrication engineers. At low sliding speeds which are representative of clinical conditions, the lubricant film may

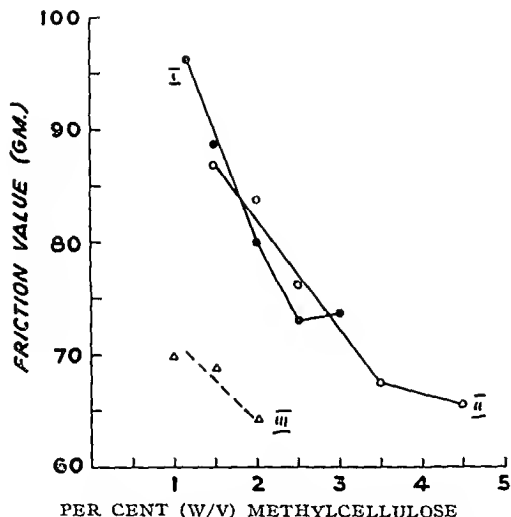


Fig. 2.—The relation of solid concentration to lubricity. ●—● Methocel 90 HG 4000; ○—○ Methocel 90 HG 400; Δ—Δ Methocel 90 HG 4000 with propylene glycol 20% v/v.

break down and a partial transition of hydrodynamic lubrication to boundary lubrication may take place. The latter is a condition where the sliding surfaces are separated by very thin lubricant films which at times may be only a few molecules thick. In this case, viscosity is less important as a determining factor for lubricity than the chemical constitution of the lubricant.

Effective boundary lubrication requires the presence of a layer of lubricant molecules on the sliding surfaces. Higher concentrations of methylcellulose in the lubricant solution should provide a more compact surface layer of the hydrocolloid. Since equal concentrations by weight of the polymer, regardless of the degree of polymerization, will provide about the same number of monomer units per volume of solution, it may be expected that equal concentrations by weight of methylcellulose, regardless of molecular weight, will exhibit a similar lubricant effect. This was found to be the case in our investigation up to the point where high viscosity interferes with lubrication.

Figure 3 represents the relation of concentration to the lubricant property of aqueous glycerin solutions. The graph shows that glycerin is an effective lubricant under the conditions tested. The lubricant properties increase with concentration, but are significant even in aqueous solutions containing only 20% glycerin. Significantly, the major increase in lubricity occurs in the lower concentration region when the viscosity changes are minor. At higher glycerin concentrations (above 50%), the changes in lubricity are not as great, while the viscosity increases profoundly.

An examination of two other polyols commonly used as components of medicinal lubricants, propylene glycol and polyethylene glycol 400, shows that their lubricant effect does not differ significantly from that of glycerin under the conditions tested. The results are tabulated in Table I.

Finally, it is of interest to determine the effect of a polyol when added to a solution of a hydrocolloid

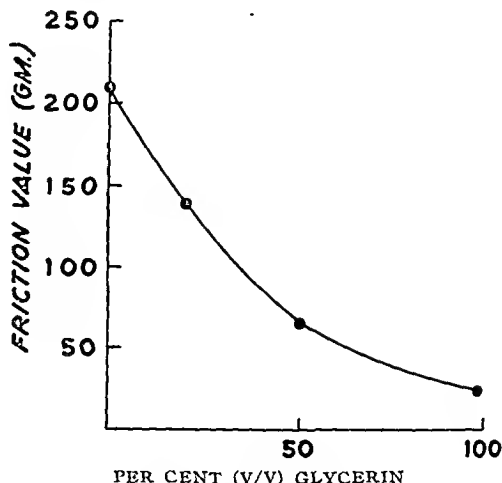


Fig. 3.—The lubricity of glycerin U.S.P.

TABLE I.—FRICTION VALUES OF POLYOLS

	U. S. P. Grade, Gm.	20% Aq. Soln., Gm.
Glycerin	22.7	138
Propylene glycol	22.0	141
Polyethylene glycol 400	17.9	130
Water <sup>a</sup>	210.0	...

<sup>a</sup> Added for comparison.

such as methylcellulose. This is shown by curve III in both Figs. 1 and 2. In both instances, regardless of whether friction value is plotted against viscosity or solid concentration, it can be seen that the lubricant effect increases significantly upon the addition of propylene glycol.

## SUMMARY AND CONCLUSIONS

1. The effect of viscosity and solid concentration on the lubricity of aqueous methylcellulose solutions was investigated with the lubrometer. The lubricating effect of selected polyols was also investigated.

2. The results indicate that the lubricating effect of methylcellulose solutions increases directly with solid concentration, while the concomitantly increasing viscosity becomes the limiting factor.

3. Glycerin, propylene glycol, and polyethylene glycol 400 are effective lubricants. Propylene glycol enhanced the lubricity of methylcellulose solutions.

## REFERENCES

- (1) Levy, G., and Schwarz, T. W., *THIS JOURNAL*, 46, 558 (1957).
- (2) Bowden, F. P., and Tabor, D., "Friction and Lubrication," John Wiley & Sons, Inc., New York, N. Y., 1956, pp. 20-22.
- (3) Norton, A. E., "Lubrication," McGraw-Hill, New York, N. Y., 1942, p. 5.
- (4) Bowden, F. P., and Tabor, D., *op. cit.*, pp. 92-93.
- (5) Petroff, N., *Eng. J.*, St. Petersburg, 1883, 71; quoted from Fuller, D. D., "Theory and Practice of Lubrication for Engineers," John Wiley & Sons, New York, N. Y., 1956.

# Ultraviolet Photometric Assay of Thiopental and Pentobarbital in Blood and Plasma\*

By STEPHEN I. OROSZLAN and GERTRUDE D. MAENGWYN-DAVIES

A procedure for the determination of sodium pentobarbital and sodium thiopental under rigidly standardized conditions of extraction, using Allen's absorption factor, is described. This method makes possible the reliable quantitative determination of 1 mcg./ml. or smaller concentration differences. In an appendix Allen's absorption factor for sodium pentobarbital is derived.

A CORRECTION FACTOR derived by Allen (1) is used routinely in the quantitative spectrophotometric estimation of steroids. This absorption factor, derived from a reading at the peak of absorption and at two wavelengths equidistant below and above the peak, can be employed when the background absorption is linear or nearly linear within the three wavelengths used.<sup>1</sup>

It was already reported by Broughton (2) that a linear absorption spectrum is obtained in blood extracts of approximately pH 13.5 (in 0.45 *N* NaOH) in the U. V. region where the barbiturates exhibit their characteristic absorption maxima at that pH. We therefore investigated whether or not a more sensitive method for the determination of pentobarbital and thiopental might be devised, under rigidly standardized conditions, employing Allen's correction factor.

## EXPERIMENTAL

### Reagents

1. NaOH stock solution, 50% (w/v). (a) NaOH, 0.45 *N*, made fresh daily from NaOH stock solution and (b) NaOH, 0.2 *N*, made fresh daily from NaOH stock solution.

2. Chloroform, reagent grade (certified, Fisher Scientific Co.), washed daily with 1 *N* NaOH and distilled water, according to Goldbaum (3).

3. NaCl, reagent grade (Merck and Co., Inc.), 0.85% solution.

4. Sodium pentobarbital (Nembutal Sodium, Abbott Laboratories) stock solution (0.4 mg./ml.) in 0.45 *N* NaOH, freshly prepared daily and kept refrigerated.

5. Sodium thiopental (Pentothal Sodium, Abbott Laboratories) stock solution (0.1 mg./ml.) in 0.2 *N* NaOH, freshly prepared daily and used immediately after preparation.

All solutions were made with glass distilled water.

### Procedure

**Preparation of Standard Curve for Sodium Pentobarbital.**—Appropriate dilutions of stock solution No. 4 were made in 0.45 *N* NaOH and read in the Beckman DU spectrophotometer in silica cells (1-cm. light path; slit width, 0.22; temperature, 22°) at three wavelengths, 245, 255 (absorption maximum), and 265 mμ. NaOH, 0.45 *N*, served as reference solution. Calculations, using Allen's (1) factor, were made as follows:

$$F = A_{255} - \frac{A_{245} + A_{265}}{2}$$

Sodium pentobarbital was determined in the range of 5 to 25 mcg./ml. The standard curve, shown in Fig. 1, was obtained by plotting *F* on the ordinate against mcg./ml. of the drug on the abscissa. Since a straight line is obtained for this plot, it obeys Beer-Lambert's law.

**Preparation of Standard Curve for Sodium Thiopental.**—This standard curve, also shown in Fig. 1, was made by appropriate dilutions of the stock solution No. 5 in 0.2 *N* NaOH, and read against 0.2 *N* NaOH as reference solution in the Beckman DU spectrophotometer under the same conditions as described above. The maximum absorption for sodium thiopental was obtained at 305 mμ, and therefore, two more readings were taken at 295 and 315 mμ. The standard curve for this drug, obtained in the range of 1 to 10 mcg./ml., was calculated as follows:

$$F = A_{305} - \frac{A_{295} + A_{315}}{2}$$

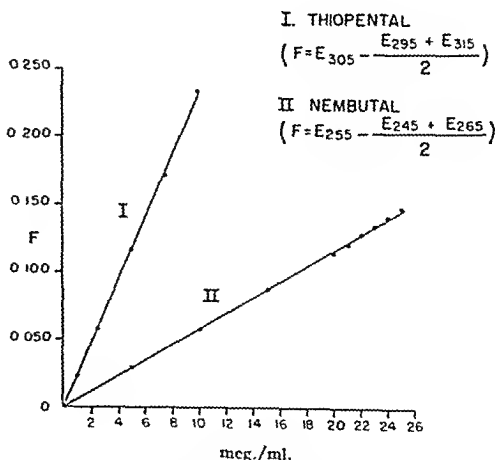


Fig. 1.—Barbiturate standard curves. Abscissa: mcg./ml. of the barbiturates. Ordinate:  $F_{\text{thiopental}}$  (I) at pH 13.2 and  $F_{\text{nembutal}}$  (II) at pH 13.45.

\* Received October 22, 1959, from Georgetown University, Schools of Medicine and Dentistry, Washington 7, D. C.  
 Aided by Grant B-1828, National Institute of Neurological Diseases and Blindness, USPHS.

<sup>1</sup> See Appendix.



As can be seen from Fig. 1, in the range studied this compound also obeyed Beer-Lambert's law, when *F* was plotted against the drug concentration (meg./ml.).

**Extraction from Whole Blood or Plasma.**—Dated plasma from the blood bank or whole oxalated dog blood was used for this purpose. Appropriate concentrations of sodium pentobarbital were dissolved in 0.85% NaCl, so that upon the addition of 0.1 ml. of the barbiturate to exactly 4.0 ml. of plasma or whole blood, a final barbiturate concentration of 50, 40, 30, and 20 meg./ml. was obtained (samples). A reference solution (blank) was prepared by adding to a 4.0-ml. aliquot of plasma or whole blood 0.1 ml. of 0.85% NaCl. Samples and blank in triplicates were extracted with 40 ml. of ehloroform in glass-stoppered bottles (125 ml.) on a variable speed shaker (Arthur H. Thomas Co.), set always at an identical slow speed setting. The bottles were shaken for exactly thirty minutes at room temperature. The speed and timing was chosen so that nearly complete extraction was obtained without the formation of emulsions. Most of the ehloroform layer was siphoned off with a glass serum lifter and filtered through Whatman No. 1 filter paper. The initial 2 ml. of the filtrate was discarded, the rest collected, and exactly 20.0 ml. of the filtrate re-extracted with 4.0 ml. of the 0.45 *N* NaOH (pentobarbital) or 0.2 *N* NaOH (thiopental) in glass-stoppered, 25-ml. eylinders by vigorous shaking by hand for one minute. After separation of the two layers, the barbiturate was determined in the clear upper layer as described for the standard

curves and read against 0.45 *N* or 0.2 *N* NaOH, respectively. The absorption of the blanks was determined to insure that the essential condition for the application of the Allen factor (1) (linearity) was met. In Table I, a reecovery experiment of triplicate extractions for whole blood is summarized.

**Comparison of the Sensivity of This Method with Known Methods.**—Sodium pentobarbital standard curves were prepared in triplicate, according to our method, Broughton's (2) method, and Goldbaum's (3) method. The results obtained are summarized in Table II.

The readings given are the arithmetic means of the triplicate determinations. The overall means and standard deviations of the means for 1 meg./ml. of sodium pentobarbital were calculated using all results obtained, and are shown in the last two lines of Table II.

## DISCUSSION

It is obvious from our results that Allen's (1) factor (*F*) can be applied to the quantitative ultraviolet determination of barbiturates. An inspection of Table II clearly indicates that with the aid of *F*, a far more reliable and sensitive method for quantitative barbiturate analysis is made possible. The error involved for the determination of 1 mcg./ml. of pentobarbital, when trying to differentiate small concentration differences, is: 10.5% by Goldbaum's method (3), 28.7% by Broughton's (2) method, 12.7% by direct alkaline U. V., and 5.4% by our method. Also, our reecovery data show an average of 99.14% by our method, and 95% by Goldbaum's (3) method, as determined by us under carefully standardized conditions of extraction procedure. We therefore believe that our method, using a rigidly standardized extraction procedure and employing Allen's (1) factor for the readings, yields a considerably more sensitive and reliable barbiturate assay. In our case we read the same final solution at three different wavelengths at the same pH. By the other methods, further dilutions and treatment of the barbiturate solutions to

TABLE I.—RECOVERY OF PENTOBARBITAL FROM BLOOD

Added, mcg./ml.	Recovered, mcg./ml.	Recovery, %
50	49.4	98.80
40	39.1	97.75
30	29.4	98.00
20	20.4	102.00

TABLE II.—COMPARISON OF PENTOBARBITAL STANDARD CURVES BY DIFFERENT U. V.-SPECTROPHOTOMETRIC METHODS

Pento- barbital Na, mcg./ml.	Our Metbod, <i>F</i>	<i>A</i> at Absorp- tion Maximum <sup>a</sup>	Goldbaum's Method (3), <i>A</i> <sub>diff.</sub> <sup>b</sup>	Broughton's Method (2), <i>A</i> <sub>diff.</sub> <sup>c</sup>
20	0.113	0.537	...	0.386
20.4	...	...	0.435	...
21	0.120	0.581	...	0.409
21.6	...	...	0.461	...
22	0.127	0.607	...	0.425
22.8	...	...	0.479	...
23	0.133	0.631	...	0.427
24	0.139	0.658	0.501	0.452
25	0.147	0.697	...	0.461
Mean increase for 1 mcg./ml. con- centration in- crement	<i>F</i> × 10 <sup>3</sup> = 6.80	<i>A</i> <sub>255 mμ</sub> × 10 <sup>3</sup> = 32.00	<i>A</i> <sub>diff.</sub> × 10 <sup>3</sup> = 18.33	<i>A</i> <sub>diff.</sub> × 10 <sup>3</sup> = 15.00
±S. E. of mean × 10 <sup>3</sup> =	±0.37	±3.98	±1.92	±4.31

<sup>a</sup> *A* = Absorbance at 255 mμ at pH 13.5 (in 0.45 *N* NaOH).

<sup>b</sup> *A*<sub>diff.</sub> (Absorbance difference) = (*A* at 260 mμ at pH 13.5) — (*A* at 260 mμ at pH 10.5).

<sup>c</sup> *A*<sub>diff.</sub> = (Absorbance at 260 mμ at pH 13.5) — (Absorbance at 260 mμ at pH 10.0).

obtain a lower pH are necessary, which might account for the increase in error.

No doubt, all barbiturates which can be determined by other methods can also be quantitated employing Allen's (1) factor in our modification of Goldbaum's (3) method. This more sensitive method may not be necessary for clinical barbiturate determinations, where such small concentration differences could be overlooked, but in the pharmaceutical assay of some preparations containing barbiturates and in the pharmacological study of barbiturate metabolism this modification might prove to be useful.

## APPENDIX

The arithmetic derivations below support the conclusions indicated by the equations which define Allen's factor (1). This factor can be used to correct for any quantitative absorption of mixtures where one component shows a linear absorption in the region where the other component has a peak, i. e., barbiturate determination in blood, the resolution of mixtures of some barbiturates, etc. The mathematical treatment is a quantitative statement of the intuitively obvious.  $(A)^B$  = absorbance of blood extract;  $(A)^N$  = absorbance of pure pentobarbital-Na;  $(A)^{BN} = (A)^N + (A)^B$  = absorbance of pentobarbital-Na extracted from blood;  $F$  = correction factor,<sup>1</sup> with subscripts  $B$ ,  $N$ ,  $BN$ , having the same meaning as the above superscripts.

With a linear background absorption (Line 1) and equidistance of  $a$  and  $c$  from  $b$ , we show two identical, rectangular triangles with the angle  $\alpha$ , therefore

$$b = a + h = c - h \quad (\text{Eq. 1})$$

and

$$2b = (a + h) + (c - h) \quad (\text{Eq. 2a})$$

or

$$b = (a + c)/2 \quad (\text{Eq. 2b})$$

therefore

$$F_B = (A)^{B_{255}} - \frac{(A)^{B_{265}} + (A)^{B_{245}}}{2} = b - \frac{a + c}{2} = 0 \quad (\text{Eq. 3})$$

$$F_N = (A)^{N_{255}} - \frac{(A)^{N_{265}} + (A)^{N_{245}}}{2} \quad (\text{Eq. 4})$$

and by substitution

$$F_N = (2b + e + g) - \frac{(a + d) + (c + f)}{2} \quad (\text{Eq. 5})$$

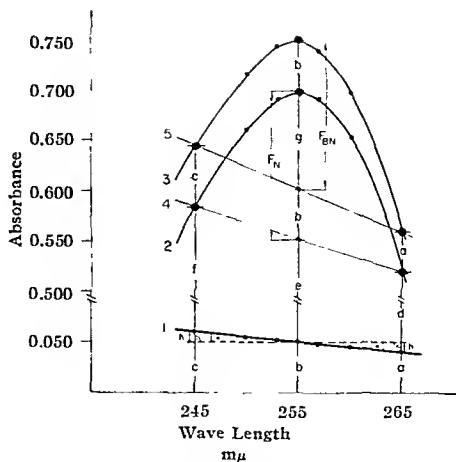


Fig. 2.—Diagram for derivation of Allen's correction factor. Line 1, background absorption of blood extract in 0.45  $N$  NaOH; curve 2, absorption of pure pentobarbital-Na (25 mcg./ml.) in 0.45  $N$  NaOH; and curve 3, absorption of pentobarbital-Na (25 mcg./ml.) extracted from blood in 0.45  $N$  NaOH.

In accordance with the derivation of Eq. 2b

$$\frac{(a + d) + (c + f)}{2} = (e + b) \quad (\text{Eq. 6})$$

therefore

$$F_N = (2b + e + g) - (e + b) \quad (\text{Eq. 7a})$$

or

$$F_N = b + g \quad (\text{Eq. 7b})$$

$$F_{BN} = \frac{(A)^{BN_{255}} - (A)^{BN_{265}} + (A)^{BN_{245}}}{2} \quad (\text{Eq. 8})$$

and by substitution

$$F_{BN} = (3b + e + g) - \frac{(2a + d) + (2c + f)}{2} \quad (\text{Eq. 9})$$

In accordance with the derivation of Eq. 2b

$$\frac{(2a + d) + (2c + f)}{2} = (2b + e) \quad (\text{Eq. 10})$$

therefore

$$F_{BN} = (3b + e + g) - (2b + e) \quad (\text{Eq. 11a})$$

or

$$F_{BN} = b + g \quad (\text{Eq. 11b})$$

From Eq. 7b and 11b

$$F_N = F_{BN} = b + g \quad (\text{Eq. 12})$$

## REFERENCES

- (1) Allen, W. M., *J. Clin. Endocrinol.*, 10, 71(1950).
- (2) Broughton, P. M. G., *Biochem. J.*, 63, 207(1956).
- (3) Goldbaum, L. R., *Anal. Chem.*, 24, 1604(1952).

<sup>1</sup> On the diagram (Fig. 2) the designation of the ordinates ( $A$ -values) are as follows:  $(A)^{B_{245}} = a$ ;  $(A)^{B_{255}} = b$ ;  $(A)^{B_{265}} = c$ ;  $(A)^{N_{245}} = d$ ;  $(A)^{N_{255}} = e$ ;  $(A)^{N_{265}} = f$ ;  $(A)^{BN_{245}} = g$ ;  $(A)^{BN_{255}} = h$ ;  $(A)^{BN_{265}} = i$ . (lines 4 and 5 cut off a distance from this ordinate which is equal to  $b$ ).

Since the absorbances of pure nembutal and the blood extract impurity are additive, it follows that:  $(A)^{BN_{245}} = 2a + d$ ;  $(A)^{BN_{255}} = 2e + g$ ;  $(A)^{BN_{265}} = 2b + f + i$ .

# Effects of Psychopharmacologic Agents on Experimentally-Induced Seizures in Mice\*

By GREGORY B. FINK† and EWART A. SWINYARD

✓ Of seven psychopharmacologic drugs screened by five assay procedures, only meprobamate and phenaglycodol exhibited significant anticonvulsant activity. Chlorpromazine, promazine, reserpine, and hydroxyzine lowered the threshold for minimal seizures variously induced, whereas triflupromazine had no effect on seizure threshold. The data obtained correlate well with available clinical data and indicate the value of threshold seizure studies in animals for detecting agents which may cause convulsions in man.

**D**ESPITE THE CONSIDERABLE attention given to both the anticonvulsant and the convulsant properties of psychopharmacologic agents, there is a lack of agreement as to the ability of these agents to alter the seizure threshold in laboratory animals and man. In laboratory animals, chlorpromazine has been reported both to lower seizure threshold (1, 2) and to exhibit anticonvulsant properties (3). In man, reserpine has been reported both to increase (4) and to decrease (5, 6) the incidence of convulsive seizures in mentally ill epileptics. Furthermore, published experimental data have been difficult to compare, because of differences in the techniques employed in various laboratories, and because the drugs under scrutiny were usually tested by only one or two assay procedures. Therefore, it was thought important to make a critical study of the effects of selected psychopharmacologic agents on experimentally-induced seizures by a battery of well-standardized methods. For comparative purposes, diphenylhydantoin and phenobarbital were also included as classical prototype anticonvulsants.

## METHODS

Male albino mice (Carworth Farms, CF No. 1 strain) weighing 22 to 36 Gm. were used as experimental animals. They were maintained on Rockland mouse diet and allowed free access to food and water except for the short time they were removed from their cages for testing. The following agents were studied: chlorpromazine hydrochloride (Thorazine), promazine hydrochloride (Sparine), triflupromazine hydrochloride (Vesprin), reserpine, hydroxyzine hydrochloride (Atarax), meprobamate (Equanil, Miltown), phenaglycodol (Ultran), phenobarbital sodium, and diphenylhydantoin sodium. All drugs were given orally in aqueous solution,

except that meprobamate and phenaglycodol were given as suspensions in 6% acacia solution.

**Anticonvulsant Assay Methods.**—Anticonvulsant potencies ( $ED_{50}$ s) were determined by a battery of four electrical tests and one chemical test. The tests based on electrically-induced convulsions measured the ability of a drug to prevent the hindleg tonic-extensor component of maximal electroshock seizures evoked by supramaximal current (MES test; 50 ma, alternating current, 0.2-second stimulus duration), to elevate the threshold for minimal electroshock seizures (alternating current, 0.2-second stimulus duration) in normal mice (a. c. EST test) and in hyponatremic (low-threshold) mice (HET test), and to elevate the threshold for low-frequency electroshock seizures (l. f. EST) induced in mice by unidirectional current delivered at an intensity twice threshold (0.2-millisecond duration, 3-second stimulus duration, six pulses per second). The test based on chemically-induced convulsions measured the ability of a drug to afford complete protection against seizures evoked by the subcutaneous injection of pentylenetetrazol (Metrazol; 85 mg./Kg.; s. c. Met. test). Except that a Grass stimulator (model S4B) was used for the l. f. EST test, the details of the various procedures, the end points employed in mice, and the characteristics of the electroshock apparatus have been described elsewhere (7-9). In addition, the mean neurotoxic dose ( $TD_{50}$ ) and the mean lethal dose ( $LD_{50}$ ) were determined for each drug. The end point for minimal neurotoxicity was muscular incoordination, based on inability of the animal to remain for one minute on a horizontal rod rotating at 5 revolutions per minute. Each drug was tested at the time of its peak activity as measured by the neurotoxicity test. For the determination of the  $ED_{50}$ ,  $TD_{50}$ , or  $LD_{50}$ , groups of six or more mice were given various doses of drug until at least three points were established in the range between 0 and 100% seizure protection, minimal neurotoxicity, or mortality. The results obtained were then plotted on logarithmic probability paper and a regression line was fitted to the plotted points by eye. From this plot of the data the respective  $ED_{50}$ ,  $TD_{50}$ , or  $LD_{50}$  was determined and the protective indexes ( $PI = TD_{50}/ED_{50}$ ) were computed; 95% fiducial limits were calculated by the method of Litchfield and Wilcoxon (10).

**Convulsant Assay Methods.**—Drugs which lacked anticonvulsant activity were examined for convulsant properties by the three electroshock threshold procedures (l. f. EST, a. c. EST, and HET) mentioned above, except that the experimental design

\* Received August 21, 1959, from the Departments of Pharmacology, University of Utah, College of Pharmacy and College of Medicine, Salt Lake City.

† This investigation was supported by a research grant from the National Institute of Neurological Diseases and Blindness (B-381), National Institutes of Health, U. S. Public Health Service.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Predoctoral trainee under National Institutes of Health Pharmacology Training Grant (2G-153).

TABLE I.—TOXICITY AND ANTICONVULSANT ACTIVITY OF SOME PSYCHOPHARMACOLOGIC DRUGS

Drug	Time of test, min.	LD <sub>50</sub> mg./Kg.	TD <sub>50</sub> mg./Kg.	ED <sub>50</sub> , mg./Kg.				
				MES	a. c. EST	HET	l. f. EST	s. c. Met.
Diphenylhydantoin	180	490 (295-808) <sup>a</sup>	84 (74 3-94 8)	14 4 (12 0-17 3)	>200	37 (24 6-55 3)	25 (16 7-37 5)	>200
Phenobarbital	180	200 (167-240)	70 (58 3-84 0)	30 (23 6-38 1)	33 (21 3-51 1)	26 (17 3-39 0)	20 (13 3-30 0)	18 5 (12.7-26.8)
Meprobamate	30	1,350 (1,205-1,512)	228 (196-262)	187 (147-237)	190 (152-238)	190 (138-260)	123 (89.8-168)	89 (73 1-119)
Phenaglycodol	60	760 (678-852)	170 (119-241)	135 (120-151)	100 (62 9-159)	80 (57 6-111)	73 (59 3-89 7)	59 (48 0-72 5)
Chlorpromazine	90	580 (460-730)	15 7 (10 6-23 2)	261 (209-326)	>200	>200	>80	>300
Promazine	60	485 (323-727)	23 5 (15 0-36 9)	175 (152-201)	>200	>150	>100	>150
Triflupromazine	90	330 (206-512)	7.8 (5 3-11.5)	>300	>200	>150	>50	>200
Reserpine	240	390 (265-573)	16.5 (11 6-23 4)	>300	>200	>200	>33	>200
Hydroxyzine	30	515 (464-571)	490 (434-553)	>350	>350	>350	>350	>350

<sup>a</sup> Values in parentheses are 95% fiducial limits.

was modified to measure seizure threshold quantitatively. In addition, pentylenetetrazol seizure threshold (i. v. Met.) was measured by the technique of Orloff, *et al.* (11), as modified by McQuarrie and Fingl (12). For each electroshock procedure, 60 or more mice were randomly divided into two equal groups; for the chemoshock procedure, 20 or more mice similarly were randomly divided into two equal groups. One group of mice was given the drug to be tested and the other (control) group was given the requisite volume of vehicle. At the previously determined time of peak drug effect, seizure threshold was determined in both groups by technique previously described (12, 13, 14). Results are presented as threshold ratios (threshold of drug-treated group/threshold of control group). Threshold ratios were determined at dose levels equivalent to fractions and multiples of the TD<sub>50</sub>s of the several drugs

## RESULTS

The time of peak effect, neurotoxicity, and anticonvulsant activity of the agents tested are summarized in Table I. The time of peak effect of the several agents varied from thirty minutes for meprobamate and hydroxyzine to as long as two hundred and forty minutes for reserpine. Phenobarbital had the lowest LD<sub>50</sub> (forty-eight-hour observation period) and the two substituted diols the highest. The LD<sub>50</sub>s of the other six drugs ranked intermediate to those for phenobarbital and the substituted diols and in most instances were not significantly different from each other. The TD<sub>50</sub>s for the nine drugs varied from 7.8 mg./Kg. for triflupromazine, the most neurotoxic, to 490 mg./Kg. for hydroxyzine, the least neurotoxic. The remaining seven compounds can be divided into the following groups ranked in order of increasing TD<sub>50</sub>s: (a) chlorpromazine, promazine, and reserpine, (b) diphenylhydantoin and phenobarbital, and (c) meprobamate and phenaglycodol. There

is no significant difference in the TD<sub>50</sub>s of the drugs within each group.

With regard to anticonvulsant activity, only phenobarbital and the two substituted diols were effective by all five tests. All three drugs were the most potent by the s. c. Met. test; the ED<sub>50</sub>s for phenobarbital, meprobamate, and phenaglycodol by this test were 18.5, 89, and 59 mg./Kg., respectively. Diphenylhydantoin exhibited the lowest ED<sub>50</sub> (14.4 mg./Kg.) in the MES test, was effective in nontoxic doses by the HET and l. f. EST tests, but was inactive by the a. c. EST and s. c. Met. tests. The remaining five drugs, chlorpromazine, promazine, triflupromazine, reserpine, and hydroxyzine, were ineffective anticonvulsants by all five tests, except that highly toxic doses of chlorpromazine and promazine (261 and 175 mg./Kg., respectively) abolished the tonic-extensor component of the MES pattern. Reserpine, in fact, increased the severity of experimentally-induced seizures, and many reserpine-treated mice died following the convulsions.

The drugs which were ineffective by the five anticonvulsant tests were studied for ability to lower the threshold for electrically and chemically-induced seizures. Threshold ratios (threshold of drug group/threshold of control group), as shown in Table II, were determined at fractions and multiples of the TD<sub>50</sub> for the phenothiazines and reserpine, and at fractions and multiples of the LD<sub>50</sub> (350 mg./Kg.) for hydroxyzine. Chlorpromazine significantly lowered l. f. EST and a. c. EST at 1/2 the TD<sub>50</sub> (7.8 mg./Kg.) and the TD<sub>50</sub> (15.7 mg./Kg.), but it did not alter the l. f. EST at twice the TD<sub>50</sub> (31.4 mg./Kg.). Promazine significantly lowered both l. f. EST and a. c. EST at all dose levels employed, except for the a. c. EST at 1/2 the TD<sub>50</sub> (11.7 mg./Kg.) the decrease was not significant; even at 4 times the TD<sub>50</sub> (94 mg./Kg.) the decrease in l. f. EST was significant. The other phenothiazine, triflupromazine, had no significant effect on electroshock seizure thresholds. Reserpine also lowered electroshock

TABLE II.—EFFECTS OF SOME PSYCHOPHARMACOLOGIC DRUGS ON THE THRESHOLD FOR ELECTRICAL AND CHEMICALLY-INDUCED SEIZURES IN MICE

Drug	Dose mg./Kg.	Threshold Ratios <sup>a</sup>			
		i. f. EST	a. c. EST	HET	i. v. Met.
Chlorpromazine	7.8	0.86 <sup>b</sup>	0.90 <sup>b</sup>	...	1.04
	15.7	0.83 <sup>b</sup>	0.84 <sup>b</sup>	0.90	0.90
	31.4	0.99	...	...	0.96
Promazine	11.7	0.85 <sup>b</sup>	0.91	...	0.80 <sup>b</sup>
	23.5	0.79 <sup>b</sup>	0.89 <sup>b</sup>	0.93	0.86 <sup>b</sup>
	47.0	0.84 <sup>b</sup>	...	...	0.90
	94.0	0.81 <sup>b</sup>	...	...	...
Triflupromazine	3.9	1.04	1.00	...	...
	7.8	0.93	1.00	0.94	1.02
	15.6	0.99	...	...	1.05
Reserpine	4.1	0.89	0.86 <sup>b</sup>	...	...
	8.2	0.71 <sup>b</sup>	0.79 <sup>b</sup>	0.93	0.91
	16.5	...	0.88 <sup>b</sup>	...	0.76 <sup>b</sup>
	33.0	...	0.80 <sup>b</sup>	...	...
Hydroxyzine	87.5	0.92	1.01	...	0.79 <sup>b</sup>
	175.0	0.96	0.93	1.04	0.86 <sup>b</sup>
	350.0 <sup>c</sup>	0.98	0.98	...	...

<sup>a</sup> Threshold of drug-treated group/threshold of control group. <sup>b</sup> Significant decrease ( $P < 0.05$ ). <sup>c</sup> LD<sub>50</sub>.

TABLE III.—PROTECTIVE INDEXES OF SOME ANTIEPILEPTIC AND PSYCHOPHARMACOLOGIC DRUGS BY FIVE ASSAY METHODS IN MICE

Drug	MES	a. c. EST	Protective Indexes <sup>a</sup>		i. f. EST	s. c. Met.
			HET			
Diphenylhydantoin	5.8	...	2.3	3.4	...	...
	(4.7-7.3) <sup>b</sup>	...	(1.5-3.5)	(2.2-5.1)	...	...
Phenobarbital	2.3	2.1	2.7	3.5	3.8	3.8
	(1.7-3.2)	(1.3-3.4)	(1.7-4.2)	(2.3-5.4)	(2.5-5.7)	(2.5-5.7)
Meprobamate	1.2	1.2	1.2	1.9	2.6	2.6
	(0.9-1.6)	(0.9-1.7)	(0.9-1.7)	(1.3-2.6)	(1.8-3.6)	(1.8-3.6)
Phenaglycodol	1.3	1.7	2.1	2.3	2.9	2.9
	(0.9-1.8)	(0.9-3.1)	(1.3-3.5)	(1.6-3.5)	(1.9-4.3)	(1.9-4.3)

<sup>a</sup> PI = TD<sub>50</sub>/ED<sub>50</sub>. <sup>b</sup> Values in parentheses are 95% fiducial limits. <sup>c</sup> Inactive by this test.

seizure thresholds at all dose levels, except that at a dose of  $\frac{1}{4}$  the TD<sub>50</sub> (4.1 mg./Kg.) the decrease in i. f. EST was not significant. In doses up to the LD<sub>50</sub> (350 mg./Kg.), hydroxyzine had no significant effect on the electroshock seizure thresholds. None of the drugs had any significant effect on the HET at the dose levels employed. Promazine was the only phenothiazine to have a significant effect on the chemoshock seizure threshold. This drug significantly lowered i. v. Metrazol threshold at 11.7 and 23.5 mg./Kg., whereas chlorpromazine and triflupromazine had no effect, even in doses twice the TD<sub>50</sub> (31.4 and 15.6 mg./Kg., respectively). Reserpine significantly reduced the chemoshock seizure threshold at the TD<sub>50</sub> (16.5 mg./Kg.) but not at  $\frac{1}{2}$  the TD<sub>50</sub>. Many reserpine-treated mice exhibited a maximal seizure immediately following the clonic seizure and expired, this rarely occurred in mice given other drugs. Hydroxyzine also lowered the i. v. Metrazol threshold in non-toxic doses of 87.5 and 175 mg./Kg.

## DISCUSSION

The data presented show a wide latitude in the LD<sub>50</sub>s and TD<sub>50</sub>s of the nine drugs studied. The LD<sub>50</sub>s indicate that phenobarbital is the most toxic and meprobamate the least toxic, when death is taken as the end point. On the other hand, the TD<sub>50</sub>s indicate that triflupromazine is the most

toxic and hydroxyzine the least toxic, when minimal overt evidence of muscular incoordination is taken as the end point. Ratios obtained by dividing the LD<sub>50</sub> by the TD<sub>50</sub> of each drug indicate that the phenothiazines and reserpine have the highest ratios; diphenylhydantoin, phenobarbital, and substituted diols have intermediate ratios; and hydroxyzine has the lowest ratio of the nine drugs studied. For example, the ratio for triflupromazine is 42.3; in marked contrast, hydroxyzine has a ratio of only 1.05. These observations emphasize that both the LD<sub>50</sub> and the TD<sub>50</sub> should be considered when estimating the toxicity of a new drug.

Only four drugs, diphenylhydantoin, phenobarbital, meprobamate, and phenaglycodol, exhibited anticonvulsant activity. Their relative anticonvulsant effectiveness, expressed as protective indexes (PI = TD<sub>50</sub>/ED<sub>50</sub>), are shown in Table III. Diphenylhydantoin has the highest protective index by the MES pattern test, but it is ineffective by two of the four threshold tests. Phenobarbital, meprobamate, and phenaglycodol are effective in nontoxic doses by all five tests; hence, they exhibit PIs greater than 1.0. Thus, in mice, the two substituted diols have margins of safety and profiles of anticonvulsant activity quite similar to those of phenobarbital. These experimental results are in agreement with clinical reports which indicate that meprobamate (15-17) and phenaglycodol (18) are of some clinical value in certain types of epilepsy.

The anticonvulsant data reported herein corroborate previous reports on the unique profile of action of diphenylhydantoin (7, 8, 19). The data also agree with our previous experiments which show that the two substituted diols resemble phenobarbital in that they modify both maximal audiogenic and maximal electroshock seizure patterns (20) and elevate the threshold for 1 f EST and i. v. Met. (13, 14, 21). Other workers have also commented on the barbiturate-like action of meprobamate (22). Several reports from our laboratories have emphasized that diphenylhydantoin is more effective in preventing seizure spread than in elevating seizure threshold, whereas phenobarbital both prevents seizure spread and elevates seizure threshold (19, 23). Since the substituted diols exhibit a profile of activity which resembles that of phenobarbital rather than diphenylhydantoin, their anticonvulsant activity would seem to be due to both mechanisms of action.

The results obtained by the convulsant assay methods agree with reported clinical observations that certain of the agents studied can induce seizures, and suggest that these tests may be of value for revealing seizure-evoking properties of new psychopharmacologic drugs. Chlorpromazine, promazine, and reserpine significantly lower both 1 f. EST and a. c. EST, whereas promazine, reserpine, and hydroxyzine lower i. v. Met. threshold; all of these agents have been reported to cause convulsions in man, to increase the frequency of seizures in epileptic patients, or to increase fatalities in patients undergoing electroconvulsive therapy (24-34). On the other hand, trifluorpromazine had no significant effect on either electroshock or chemoshock seizure thresholds; in so far as we are aware, this particular agent has not been reported to cause seizures in man.

### SUMMARY

Seven psychopharmacologic and two anti-epileptic agents were screened by a battery of five anticonvulsant assay procedures, and the drugs found ineffective were subjected to seizure threshold studies to determine whether they had potential convulsant properties. The mean neurotoxic dose and the mean lethal dose of each drug were determined. Meprobamate and phenaglycodol were effective in nontoxic doses by all five anticonvulsant tests and exhibited a profile of activity which resembled that of phenobarbital. Chlorpromazine lowered the threshold for alternating current and low-frequency electroshock seizure threshold (a. c. and 1 f. EST); promazine lowered not only the a. c. and 1 f. EST but also the intravenous pentylenetetrazol threshold (i. v. Met.); trifluorpromazine had no effect on these experimental seizure thresholds. Reserpine de-

creased all three types of seizure thresholds and facilitated convulsive activity. Hydroxyzine had no effect on electroshock seizure thresholds but lowered the i. v. Met. threshold. It was concluded that the several tests employed have only limited value for screening potential psychopharmacologic agents, but that they are definitely useful for disclosing their neuropharmacological properties and for detecting any propensity to induce seizures in man.

### REFERENCES

- (1) Heming, A. E., Holtkamp, D. E., Huntsman, D. B., Doggett, M. C., and Mansor, L. F., *J. Pharmacol. Exptl. Therap.*, 116, 28 (1956).
- (2) Tedeschi, D. H., Benigni, J. P., Elder, C. J., Yeager, J. C., and Flanagan, J. V., *ibid.*, 123, 35 (1958).
- (3) Schallek, W., Kuehn, A., and Seppelin, D. K., *ibid.*, 118, 139 (1956).
- (4) Barsa, J. A., and Kline, N. S., *Arch. Neurol. Psychiat.*, 74, 31 (1955).
- (5) Brooks, C., *Diseases of Nervous System*, 18, 275 (1957).
- (6) Rosenberg, C. M., *Ohio State Med. J.*, 53, 405 (1957).
- (7) Swinyard, E. A., Brown, W. C., and Goodman, L. S., *J. Pharmacol. Exptl. Therap.*, 106, 319 (1952).
- (8) Brown, W. C., Schiffman, D. O., Swinyard, E. A., and Goodman, L. S., *ibid.*, 107, 273 (1953).
- (9) Woodbury, L. A., and Davenport, V. D., *Arch. intern. Med.*, 62, 272 (1952).
- (10) Mosier, J. M., and Wilcoxon, F., *J. Pharmacol.*, 11, 101 (1952).
- (11) Orloff, M. J., Williams, H. L., and Pfeiffer, C. C., *Proc. Soc. Exptl. Biol. Med.*, 70, 254 (1949).
- (12) McQuarrie, D. G., and Fingl, E., *J. Pharmacol. Exptl. Therap.*, 124, 264 (1958).
- (13) Swinyard, E. A., Chin, L., and Fingl, E., *Science*, 125, 739 (1957).
- (14) Chin, L., and Swinyard, E. A., *THIS JOURNAL*, 48, 6 (1959).
- (15) Perlstein, M. A., *J. Am. Med. Assoc.*, 161, 1040 (1956).
- (16) Ivanov, A. A., *N. Y. State J. Med.*, 58, 2529 (1958).
- (17) L. A. M., *A. J. Diseases*, 1, 1 (1958).
- (18) Mosier, J. M., *Proc. Soc. Exptl. Biol. Med.*, 108, 168 (1953).
- (19) Fink, G. B., and Swinyard, E. A., *ibid.*, 127, 318 (1959).
- (20) Chin, L., and Swinyard, E. A., *Proc. Soc. Exptl. Biol. Med.*, 97, 251 (1958).
- (21) Pfeiffer, C. C., Riopelle, A. J., Smith, R. P., Jenney, E. H., and Williams, H. L., *Ann. N. Y. Acad. Sci.*, 67, 734 (1957).
- (22) Toman, J. E. P., and Goodman, L. S., *Physiol. Rev.*, 28, 409 (1948).
- (23) Barrett, O., Jr., *J. Am. Med. Assoc.*, 166, 1986 (1958).
- (24) Gunter, M. J., *Ohio State Med. J.*, 54, 51 (1958).
- (25) Hankoff, L. D., Kaye, H. E., Engelhardt, D. M., and Freedman, N., *N. Y. State J. Med.*, 57, 2967 (1957).
- (26) Fazekas, J. F., Shea, J. G., Ehrmantraut, W. R., and Alman, R. W., *J. Am. Med. Assoc.*, 165, 1241 (1957).
- (27) Kinross-Wright, V. J., and Morrison, S. B., *J. Clin. Exptl. Psychopathol. & Quart. Rev. Psychiat. Neurol.*, 19, 219 (1958).
- (28) Kurtzke, J. F., *J. Nervous Mental Disease*, 125, 119 (1957).
- (29) Voegelé, G. E., and May, R. H., *Am. J. Psychiat.*, 113, 655 (1957).
- (30) Brunton, A. M., "Psychotropic Drugs," Ed. by Garattini, S., and Ghetti, V., Elsevier Publishing Co., Amsterdam, 1957, p. 405.
- (31) Shaw, E. B., *Pediatrics*, 22, 175 (1958).
- (32) Foster, M. W., Jr., and Gayle, R. F., Jr., *J. Am. Med. Assoc.*, 159, 1520 (1955).
- (33) Kalinowsky, L. B., *Am. J. Psychiat.*, 112, 745 (1956).

# The Facilitative Action of Reserpine on Metrazol Convulsions When Modified by Iproniazid\*

By L. R. WEISS†, J. W. NELSON, and A. TYE

The effects of reserpine, iproniazid, and their combination on the convulsive threshold, facilitative action, and seizure pattern of metrazol-treated mice was investigated. The effect of reserpine on metrazol convulsions is a marked decrease in convulsive dose which is accompanied by a reduced seizure latency time in the convulsive pattern with an increasing facilitative action. Iproniazid was found to increase the convulsive dose and lengthen the latency interval. Prior treatment with iproniazid before reserpine reversed the depressive state produced by the latter agent to that of stimulation. This was accompanied by an increased anticonvulsant effect, longer latency interval, and absence of a facilitative action. Treatment with iproniazid after reserpine failed to elicit these effects. These results are compatible with the hypothesis that iproniazid and reserpine produce their effect through a biochemical change, the former by monoamine oxidase inhibition and the latter by release of norepinephrine and other amines from their storage sites in the central nervous system.

THE EFFECTS of reserpine on metrazol shock in mice were reported by Chen and Bohner (1). They found that pretreatment with reserpine modified the convulsive pattern by shortening the interval between the first sign of clonic movements and the final tonic extension of the hindlimbs in mice. This interval is termed the seizure latency time in the convulsive pattern. Another observation was the decrease in the convulsive threshold which is manifested by a reduction in the dose of metrazol required to produce a comparable response. This reduced shock threshold and decreased seizure latency period appears to be a characteristic of reserpine. The central depression seen after reserpine administration, therefore, possesses a convulsive component—a contradiction to the classical anticonvulsant action of many central depressants. Moreover, with a general depression of motor activity accompanied by sedation, a marked potentiating effect on the hypnotic action of barbiturates and other central depressants is commonly seen (2). The nature of this dual mechanism involving both hypnotic potentiation and convulsive phenomena is still not clear. The current belief is that various indole and catecholamines which are found in the biochemical and metabolic pathways within the central nervous system are influenced by the action of reserpine (3, 4). The varied actions of reserpine are believed to be associated with its ability to decrease the content of primary amines (norepinephrine, dopamine, and 5-hydroxytryptamine) in the central nervous system.

The use of iproniazid, a monoamine oxidase inhibitor, as a tool for investigations in the area of brain chemistry has helped to clarify the mode by which reserpine may affect the function of the central nervous system. It is believed that it will elevate the content of primary amines in the brain (5). Combinations of iproniazid and reserpine can produce central stimulation which is characterized by an increase in sympathetic tone, tremors, and marked excitation very similar to an amphetamine-like action. This phenomenon is dependent upon the order in which iproniazid and reserpine are administered and usually appears only when iproniazid is given before reserpine. Indeed, prior treatment with iproniazid prevents the stupor and depressed state produced by reserpine in mice and other animals (6). Iproniazid has been described as a psychic stimulant but, like reserpine, it will increase the sleeping time of barbiturates (7-9). It has been reported to have anticonvulsant properties and will increase the convulsive threshold of metrazol shock (10).

These effects indicate a complex interplay between central activity and the actions which characterize iproniazid and reserpine administration. The duration of action of each drug is long lasting, seemingly irreversible, and apparently noncompetitive with respect to the different possible mechanisms of action (11, 12). The activity of these drugs persists considerably beyond the time they can be detected in the tissues of the brain, blood, and other organs, indicating that they produce some unusual change (13).

The value of metrazol shock for the routine screening of anticonvulsant agents has been firmly established (14-17). As usually employed

\* Received August 21, 1959, from the College of Pharmacy, The Ohio State University, Columbus.

† Fellow of the American Foundation for Pharmaceutical Education, 1959-1960.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

this method is based on measuring the ability of a drug to raise or lower the chemoshock threshold rather than an alteration in the seizure pattern. Recently, Toman, *et al.* (18), evaluated the seizure latency interval in the convulsive shock pattern as a screening method for investigating antipsychotic drugs. A modification of this procedure using metrazol shock has been adapted to this problem.

The convulsive pattern of metrazol seen after a challenging dose given intraperitoneally can be divided into three stages as described by Orloff, *et al.* (14). First, a sharp twitching of the animal body which is followed by a series of clonic movements and then a stage of depression or a resting phase (seizure latency interval). Finally, a persistent convulsion occurs which consists of a tonic flexor component followed immediately by a usually lethal tonic extension of the hindlimbs. The three stages follow each other as the concentration of metrazol is gradually increased in the blood stream. The ability of a compound to prevent or facilitate the convulsion can be measured in terms of the final maximal extension as the end point and the latency time as the interval between clonic and tonic signs. Reserpine does not influence the sequence of events in this convulsive pattern. It will modify the resting stage, which is considered to be the time taken for selective high frequency stimulation of the motor cortex causing localized repetitive discharges to spread out to a generalized maximal tonic seizure. It could also be considered a period of inhibition of midbrain centers or other areas holding the medullary extensor facilitating center in abeyance (18). Central depressants and adrenergic stimulating drugs will generally suppress the tonic extensor phase; barbiturates and amphetamine-like agents, and anticonvulsants raise the threshold for this convulsive discharge. On the other hand, reserpine is found capable of facilitating the tonic component, reducing the convulsive threshold, and shortening the seizure latency time.

In view of the evidence that a relationship exists between the action of iproniazid and reserpine on the various biochemical changes produced by these drugs in the central nervous system especially when coupled with the many pharmacologic effects associated with their single and combined use, an investigation concerned with the effects of reserpine and iproniazid on the convulsive threshold, changes in facilitation, and in the seizure pattern of metrazol shock is reported and discussed.

## EXPERIMENTAL

The experimental determinations were divided into three series depending on the drugs used (iproniazid, reserpine, and iproniazid-reserpine combinations), and was subdivided by various pretreatment schedules. Each series consisted of young adult female Swiss mice obtained from Maxfield farms weighing  $20 \pm 2$  Gm. All animals were kept under uniform laboratory conditions and given free access to Purina chow and water. The animals were starved and housed in isolation cages for eight hours prior to metrazol shock. An experimental series consisted of 48 animals divided into four groups of 12 mice, all pretreated in a similar manner with iproniazid, reserpine, or a combination of both drugs. In each series for each pretreatment schedule the relative increase or decrease in the shock threshold was compared to a control-convulsive dose of metrazol obtained graphically by probit method using four dosage levels representing 12 animals per dose (19). This convulsive dose of metrazol is the index that will produce the tonic hindlimb extensor component of the seizure in 50% of the treated animals ( $CD_{50}$ ). A 95% confidence limit is given with the  $CD_{50}$  of metrazol for each determination. For each group of 48 animals (four doses, 12 animals per dose) the seizure latency time was concurrently measured in those animals reaching the tonic extensor end point of the seizure pattern and was measured as the time, in seconds, between the clonic and maximal tonic phases. The mean time of those animals reaching the tonic extensor stage was calculated and a 95% confidence limit was statistically established using the standard error of this mean. The time interval between the administration of metrazol and the onset of the clonic stage was found to be appreciably uniform with the control. Facilitation was seen only in the series of animals pretreated with reserpine and was evaluated as the per cent of animals in which the resting phase was absent and the convulsion seizure passed directly from a clonic to a tonic phase in each determination.

The dose of iproniazid phosphate was 150 mg./Kg. given i. p. at various pretreatment intervals. This dose is nontoxic, produced few positive observable effects in mice after five days of daily administration, and was established as that dose which will inhibit monoamine oxidase and cause reserpine reversal.

The dose of reserpine was 8 mg./Kg. given i. p. at various pretreatment intervals with iproniazid. When given alone the dose was varied as indicated below.

The pretreatment schedule used with each drug or combination of drugs was based on reports by other investigators and partially on preliminary findings in our laboratory. Iproniazid (150 mg./Kg.) was injected at four, eight, twelve, and twenty-four hours, and daily for two, three, four, and five days prior to metrazol shock. In the latter grouping, shock was given twenty-four hours after the last treatment. Reserpine (8 mg./Kg.) was injected four and eight hours before shock; 4.0, 2.0, 1.0, 0.1 mg./Kg. was injected four hours before shock; and 0.1 mg./Kg. was given daily for ten days, the last dose being twenty-four hours before metrazol shock. Combinations of iproniazid (150 mg./Kg.) and reserpine (8 mg./Kg.) were injected at various in-



tervals before metrazol shock. These pretreatment combinations were iproniazid and reserpine together four hours before; reserpine (eight hours) and iproniazid (four hours) before; iproniazid (eight hours) and reserpine (four hours) before; iproniazid (twenty-four hours) and reserpine (four hours) before; iproniazid (daily for two days) and reserpine (four hours) before; iproniazid (daily for three days) and reserpine (four hours) before; iproniazid (daily for four days) and reserpine (four hours) before; iproniazid (daily for five days) and reserpine (four hours) before metrazol shock.

## RESULTS AND DISCUSSION

This investigation is primarily concerned with the effects of iproniazid and reserpine on the convulsive threshold, facilitative action, and seizure latency time. It also compares the relative changes in the convulsive dose ( $CD_{50}$ ) and the seizure pattern under similar pretreatment intervals and doses for iproniazid and reserpine. The results of this study are grouped according to the pretreatment schedule of drugs. Figure 1 indicates the responses seen with iproniazid pretreatment. Each set of adjacent bars represents two different parameters, the  $CD_{50}$  (mg./Kg.) and the seizure latency time (seconds). Figure 2 shows the effects seen with reserpine. The facilitative action of this drug is shown as the per cent of animals reaching the final tonic end point without showing the characteristic latency period of the convulsive seizure. Figure 3 represents the combined pretreatment with iproniazid and reserpine. In this figure three situations occurred where the facilitative action of reserpine was observed. The importance of this effect will be discussed below. The net result shown in Fig. 3 is that the optimal response in Fig. 2, e. g., reserpine (8.0 mg./Kg.) four hours, has been superimposed upon the effects of iproniazid seen in Fig. 1, making the various interactions of these drugs stand out as a function of the pretreatment interval which was used with each drug before metrazol shock.

In Fig. 1, pretreatment with iproniazid in single doses showed a moderate increase in the  $CD_{50}$  as the pretreatment interval was lengthened from four to twenty-four hours. This could be due to the effect of inhibiting the enzyme, monoamine oxidase, and the accompanying rise in the level of norepinephrine and other amines. Daily doses of iproniazid for two, three, four, and five days did not appreciably change the elevated convulsive threshold and the anticonvulsive effect. This would indicate the maximal action of this drug on metrazol shock was reached. The seizure latency time was similar or raised above the control index in all of the pretreatment periods. The gross effect was a general lengthening of the resting stage of the convulsive pattern with iproniazid pretreatment. In summary, the results of this series suggest that the convulsive dose and seizure latency time, while apparently unrelated, seem to provide a more accurate picture of the total anticonvulsant effect of iproniazid.

In Fig. 2 the effects of various doses of reserpine (0.1 to 8.0 mg./Kg.) using pretreatment intervals of four and eight hours and a ten-day period of daily doses (0.1 mg./Kg.) are shown together with the influence of this drug on the convulsive dose, facilitatory

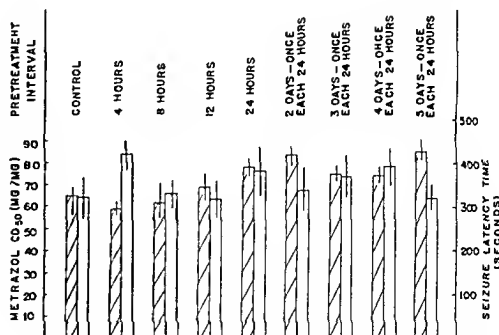


Fig. 1.—The effect of iproniazid on the convulsive dose and seizure latency time of metrazol. Pretreatment with iproniazid (150 mg./Kg.)  $CD_{50}$  [hatched], latency [white].

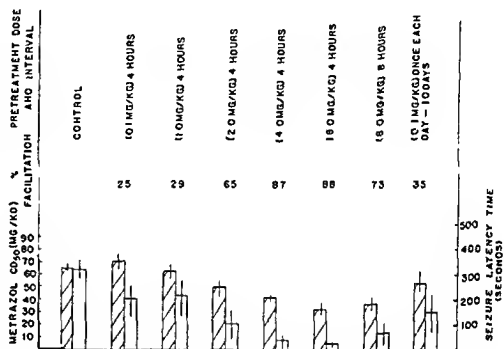


Fig. 2.—The effect of reserpine on the convulsive dose, seizure latency time, and facilitative action of metrazol.  $CD_{50}$  [hatched], latency [white], facilitation [dotted].

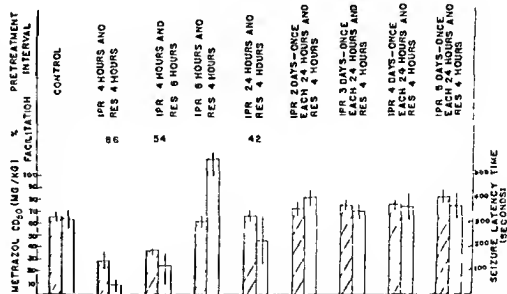


Fig. 3.—The effect of iproniazid and reserpine on the convulsive dose, seizure latency time, and facilitative action of metrazol. Pretreatment with iproniazid (150 mg./Kg.) and reserpine (8 mg./Kg.)  $CD_{50}$  [hatched], latency [white], facilitation [dotted].

action, and the seizure latency time. In sharp contrast to that of iproniazid, the relative change in the convulsive threshold and latency interval seems to depend on the change in the dose of reserpine. High doses (2.0 to 8.0 mg./Kg.) lowered the convulsive dose almost 50% and the seizure latency time dropped 90% below the control level. The results tend to show a correlation between the dose of reserpine and the magnitude of the response, as can be observed by the steady fall in  $CD_{50}$  and latency time

as the dose of reserpine was increased from 0.1 to 80 mg./Kg. This relationship was also noticed in the proportion of animals showing no resting period, going from initial clonic to final tonic convulsion with increasing doses of reserpine. Extending the pretreatment interval or reducing the dose decreased the facilitating effect, raised the  $CD_{50}$ , and lengthened the latency time. Prolonged administration of 0.1 mg./Kg. of reserpine caused a moderate decrease in the  $CD_{50}$  and latency time with some increased facilitative action, perhaps due to the cumulative and prolonged effects of this drug. Summarizing the action of reserpine on metrazol shock; facilitation, latency time, and the convulsive threshold appear to be a function of the dose of reserpine, the former effect changing directly and the latter two changing inversely with the dose of reserpine. With this drug, the latency interval and the threshold of stimulation appear to complement each other as the amount of facilitation is increased. These findings with reserpine are in agreement with those of other investigators (20, 21).

In Fig. 3, the combined effect of iproniazid and reserpine is pictured as a predominating action of the drug which was administered first and the pretreatment interval between these drugs. Essentially, prior treatment with reserpine at four and eight hours gives much the same results noted in Fig. 2 for the same dosage and time. The anticonvulsant properties of iproniazid did not seem to reverse the low convulsive threshold, short latency time, and facilitatory action to any appreciable extent. It was observed that all the animals first treated with reserpine were characteristically depressed and remained in this state after iproniazid was given. In marked contrast to these findings are those seen with iproniazid treatment before reserpine. Reference to Fig. 1 shows a striking similarity between the prior treatment with iproniazid alone and when reserpine is given after iproniazid, as observed in Fig. 3. These animals were stimulated and active approximately a half hour after reserpine was added to their schedule. This adrenergic-type reaction was not apparent in those animals treated with iproniazid alone, as in Fig. 1. Consequently, an anticonvulsant effect was noted with the iproniazid-induced reversal of reserpine stupor. Apparently the protection by iproniazid was diminished after twenty-four hours as indicated by the lowered latency time and increased facilitative action. However, reinforcement with daily doses of iproniazid restored the anticonvulsant effect. The seizure latency time was lengthened and a general increase in the convulsive threshold was observed. The finding that iproniazid has anticonvulsant properties agrees with reports that various monoamine oxidase inhibitors, including iproniazid, protect against maximal shock seizures (22). The onset and duration of this agent seems to coincide with the reported rapid and persistent ability to in-

crease brain levels of norepinephrine and 5-hydroxytryptamine (23).

Pretreatment with reserpine will lower the level of these amines. This would suggest that the anticonvulsant effect of iproniazid and reserpine combinations are the result of an increased level of amines caused by pretreatment with iproniazid before reserpine which prevents the destruction of those amines released by reserpine. It follows that the latent convulsive effect of reserpine is a reflection of the release, inactivation, and lowering of the amine level in the brain.

While our data do not directly support the relative importance of monoamine oxidase inhibition and the level of norepinephrine and other amines in the convulsive phenomenon, the results observed in this study on the convulsive dose, seizure latency time, and facilitative action of iproniazid and reserpine reflect many of the changes produced by these drugs. Although this would point to these biochemical factors as controlling the neurological mechanism, convulsive seizure and the related convulsive threshold it appears very unlikely that the anticonvulsant and convulsant properties of the many drugs used today all depend on the same factors.

## REFERENCES

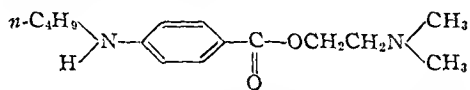
- (1) Chen, G., and Bohner, B., *J. Pharmacol. Exptl. Therap.*, **116**, 12 (1956).
- (2) Shore, P. A., Silver, S. H., and Brodie, B. B., *Science*, **122**, 248 (1958).
- (3) Brodie, B. B., and Shore, P. A., *Ann. N. Y. Acad. Sci.*, **66**, 631 (1957).
- (4) Brodie, B. B., Spector, S., and Shore, P. A., *Pharmacol. Rev.*, **11**, 548 (1959).
- (5) Symposium on the Biochemical and Clinical Aspects of Mersilid and other Monoamine Oxidase Inhibitors, *J. Clin. Exptl. Psychopath.*, **19**, Suppl. 1 (1958).
- (6) Chessin, M., Kramer, E. R., and Scott, C. C., *J. Pharmacol. Exptl. Therap.*, **119**, 453 (1957).
- (7) Brodie, B. B., Pletscher, A., and Shore, P. A., *ibid.*, **116**, 84 (1955).
- (8) Zeller, E. A., Barsky, J., Fouts, J. R., and Lazanas, J. C., *Biochem. J.*, **60**, 5 (1955).
- (9) Hess, S. W., Weissbach, H., Redfield, M., and Udenfriend, S., *J. Pharmacol. Exptl. Therap.*, **118**, 84 (1956).
- (10) Hess, S. W., Weissbach, H., and Udenfriend, S., *ibid.*, **124**, 189 (1958).
- (11) Pletscher, A. P., Shore, P. A., and Brodie, B. B., *Science*, **122**, 374 (1955).
- (12) Davison, A. N., *Biochem. J.*, **67**, 316 (1957).
- (13) Brodie, B. B., Pletscher, A., and Shore, P. A., *J. Pharmacol. Exptl. Therap.*, **116**, 9 (1955).
- (14) Orloff, M. S., Williams, H. L., and Pfeiffer, C. C., *Proc. Soc. Exptl. Biol. Med.*, **70**, 254 (1949).
- (15) Swinyard, E. A., Brown, W. C., and Goodman, L. S., *J. Pharmacol. Exptl. Therap.*, **106**, 319 (1952).
- (16) Goodman, L. S., Toman, J. E. P., and Swinyard, E. A., *Am. J. Med.*, **1**, 213 (1946).
- (17) Chen, G., Ensor, C. R., and Bohner, B., *Proc. Soc. Exptl. Biol. Med.*, **87**, 334 (1954).
- (18) Toman, J. E. P., and Everett, G. M., "Tranquillizer Drugs, A Symposium," AAAS, Washington, D. C., Publication No. 46, 1957, pp. 23-33.
- (19) Litchfield, J., and Wilcoxon, F., *J. Pharmacol. Exptl. Therap.*, **95**, 99 (1949).
- (20) Chen, G., Ensor, C. R., and Bohner, B., *Proc. Soc. Exptl. Biol. Med.*, **85**, 507 (1954).
- (21) Jenney, E. H., *Fed. Proc.*, **13**, 370 (1954).
- (22) Prockop, D. J., Brodie, B. B., and Shore, P. A., Fall meeting 1958, *Am. Soc. Pharmacol. Exptl. Therap. Abstr.*, p. 28.
- (23) Sjoerdsma, A., Smith, T. E., Stevenson, T. D., and Udenfriend, S., *Proc. Soc. Exptl. Biol. Med.*, **89**, 36 (1955).

# Metal Complexes of Tetracaine Hydrochloride and Related Local Anesthetics\*

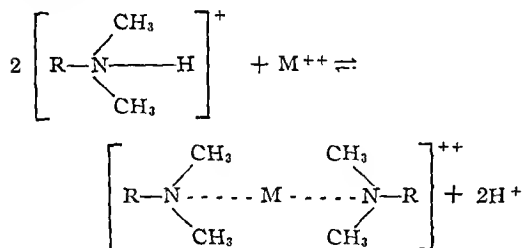
By HERBERT A. PLATT† and ALFRED N. MARTIN

The plan of this investigation was to complex tetracaine HCl and some related local anesthetics with certain metal ions; and to determine the most acceptable complexing metal ions, the stability constants of the metal complexes under varying conditions, and the biological significance of such complexes.

WITH THE POSSESSION of functional groups such as the secondary aromatic amine, the tertiary aliphatic amine, and the carboxyl group bound in the ester linkage, tetracaine has potential coordination centers.



The expectation was that tetracaine HCl should complex with suitable metal ions in the following manner:



This complexation is somewhat similar to that of metals with amino acids.

Since cations of the transition and near-transition metals have the greatest tendency for combining with electron donors (1), the following ions were selected: copper (II), nickel (II), cobalt (II), manganese (II), and zinc (II).

A rapid qualitative and quantitative method of testing for complexation is the pH effect. Qualitatively, the method is both quick and simple. A drop in pH results when the protonated form of the complexing agent forms a coordination compound with metal ions. The greater the tendency for the metal to combine with a given coordinating agent, the greater the drop in pH.

Quantitatively, it is probably the most accurate and reliable method for the determination of the stability constants (2, 3).

## EXPERIMENTAL

**Materials.**—Tetracaine HCl (U. S. P., Sterling-Winthrop Research Institute); procaine HCl (U. S. P., Mallinckrodt Chemical Works); butethamine HCl (N. F., Novocol Chem. Mfg. Co.); glycine (General Biochemicals Inc.); DL- $\alpha$ -alanine (General Biochemical Co.);  $\beta$ -alanine (General Biochemicals Inc.); and DL-serine (Nutritional Biochemicals Co.) were evaluated chromatographically and were found to be of a high degree of purity and could be used without prior treatment.

Analytical reagent grades of the metal chlorides were satisfactory and were used without further purification.

The local anesthetics, procaine HCl and butethamine HCl, in addition to tetracaine HCl, provided a parallel in the relationship between the stability constants and the structure of the local anesthetics.

The amino acids were selected to serve as reference compounds for the procedures developed and to provide a comparison between the stability constants and the structure.

**Procedure.**—Using the primary standard, potassium acid phthalate, standard sodium hydroxide solutions of approximately 0.2 *N* were prepared as needed in the usual quantitative manner.

The following is a general outline of the procedure used: a ligand hydrochloride or amino acid stock solution was freshly prepared containing approximately  $5.0 \times 10^{-4}$  moles/ml. Five milliliters, by pipet, of this stock solution ( $2.5 \times 10^{-3}$  moles) was mixed with sufficient distilled water to give 75 ml. and was titrated with the standard sodium hydroxide (0.50-ml. increments), by buret. The pH, at each addition of the base, was determined with a Beckman model GS pH meter with fiber-type calomel reference and glass (heavy duty) electrodes. One milliliter, by pipet, of the metal stock solution ( $1$  to  $10 \times 10^{-4}$  moles), also diluted to 75 ml., was titrated with sodium hydroxide.

To test for complexation with the metal, 1.00 ml. of the metal stock solution was added to 5 ml. of the ligand stock solution (all samples were diluted to 75 ml.) and the resulting solutions were titrated as outlined. In certain cases, to prevent hydroxide formation before the titration could be carried out (zinc), the titrated samples were previously acidified by the addition of 5.00 ml. of approximately 0.01 *N* HCl. The titrations were conducted in a water bath at  $30^\circ \pm 0.5^\circ$ ; the solutions were allowed to reach this temperature for fifteen to twenty minutes prior to the titration. During the titration, the solution was agitated by a slightly flattened glass stirring rod driven by an air stirrer. The agitation was not severe enough

\* Received August 21, 1959, from Purdue University, School of Pharmacy, West Lafayette, Ind.

This work was supported by a grant from the U. S. Medical Procurement Agency.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: Julius Schmid, Inc., New York, N. Y. The authors are indebted to W. H. Golod for his technical assistance in the biological portion of this study.

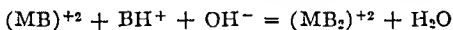
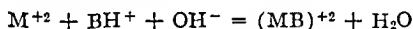
to break the surface of the liquid. The pH meter was standardized with a buffer solution of pH 7.00. All values reported are an average of two separate determinations. The precision of the pH measurements was  $\pm 0.02$  pH unit.

All calculations are based on a constant volume of 75 ml. Disregarding the volume changes, this led to an estimated error of 2% in addition to the experimental error of about 2%.

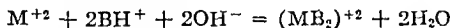
**Treatment of Data.—Qualitative.**—Applying Bjerrum's method (4) to a case of equilibrium between metal ions and a complexing agent, the following examples are presented for the pH titration of glycine (Fig. 1) and tetracaine HCl (Fig. 2) in the presence and absence of cupric ions. It was not found necessary to continue the titration to the equivalence point; each curve was carried approximately to the half-neutralization point.

Curve I, in Figs. 1 and 2, shows the titration curve of a 75-ml. sample of ligand. Curve II shows the titration curve of a 75-ml. sample of the ligand to which a smaller quantity of cupric ion was added. The curve with the metal lies below the ligand curve indicating that complexation has taken place. The first flat portion of Curve II represents the formation of the complex, the sharp rise indicates the completion of the complex formation, and the last flat portion represents the titration of the excess ligand.

**Calculation of Stability Constants.**—Using the method of Bjerrum (4), as modified by Calvin and Melchior (5),  $\bar{n}$ , the average number of ligand molecules bound to a single metal ion, may be evaluated from the horizontal distance between titration curves I and II, Figs. 1 and 2. This is exactly the amount of base consumed in the reactions, and equals the concentration of  $BH^+$  bound in the complex



and the overall reaction is



where  $M^{+2}$  represents the metal ion,  $BH^+$  is the protonated ligand, and  $(MB)^{+2}$  and  $(MB_2)^{+2}$  are complexed forms of the ligand. It is assumed that these are essentially the only reactions resulting from the uptake of base since the complexing agent is present in considerable excess with respect to the metal ion. The total moles of the ligand B bound, equivalent to the horizontal distance between curves I and II, when divided by the total concentration of metal, gives the value of  $\bar{n}$ . When  $\bar{n}$  becomes constant, the maximum for the average number of ligand molecules bound to a single metal ion is had. At any pH, the value of unbound or free B is calculated from  $p(B) = -\log B = pK_a - pH - \log [(BH^+)_{initial} - (NaOH)]$ . In doing so, a series of values of  $\bar{n}$  and B are obtained (6). Figures 3 and 4 are graphical representations of  $\bar{n}$  plotted against the negative logarithm of B, written  $p(B)$ . Approximate values for  $1/2 \log K$ , where  $\log K$  is the overall stability constant, may be read directly from these formation curves at  $\bar{n} = 1.0$ . The data are shown in Table I.

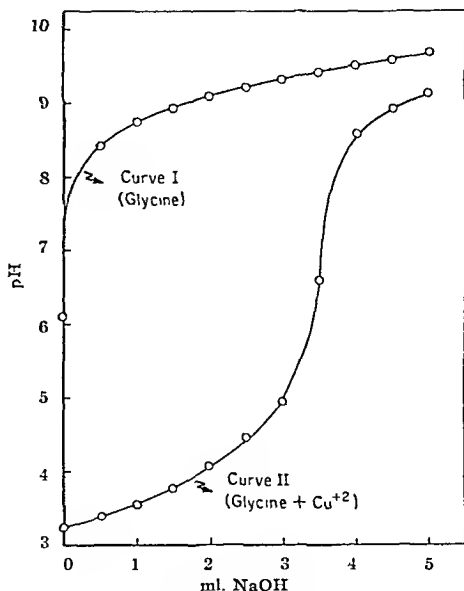


Fig. 1.—Glycine; pH titration curve in the presence and absence of cupric ions.

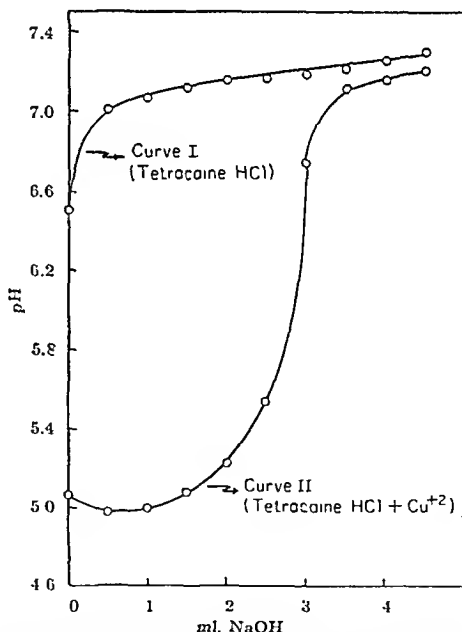


Fig. 2.—Tetracaine hydrochloride; pH titration curve in the presence and absence of cupric ions.

## RESULTS

Qualitatively, cobalt, manganese, and zinc ions did not complex with tetracaine HCl since no pH lowering was noted when tetracaine HCl was titrated in the presence of these metal ions. Nickel complexed to a very limited extent with the ligand, tetracaine.

Copper showed a tendency toward complexation with tetracaine. Since the copper-tetracaine titra-

TABLE I.—TYPICAL CALCULATION OF  $\bar{n}$  AND THE STABILITY CONSTANT

pH	H <sub>3</sub> O <sup>+</sup>	NaOH, ml	Moles OH <sup>-</sup> = moles B (bound)	$\bar{n}$	(B)	p(B)
Glycine, 30°, 75-ml. sample, $3.33 \times 10^{-2}$ moles/L.; Cu <sup>++</sup> , $6.06 \times 10^{-3}$ moles/L.; NaOH, $2.59 \times 10^{-4}$ moles/ml., 0.50-ml. increment						
3.50	$3.16 \times 10^{-4}$	0.98	$2.53 \times 10^{-4}$	0.56	$1.95 \times 10^{-8}$	7.71
4.00	$1.00 \times 10^{-4}$	1.88	$4.86 \times 10^{-4}$	1.07	$5.50 \times 10^{-8}$	7.26
4.50	$3.16 \times 10^{-5}$	2.52	$6.53 \times 10^{-4}$	1.43	$1.59 \times 10^{-7}$	6.80
5.00	$1.00 \times 10^{-5}$	3.02	$7.83 \times 10^{-4}$	1.72	$4.68 \times 10^{-7}$	6.33
5.50	$3.16 \times 10^{-6}$	3.27	$8.48 \times 10^{-4}$	1.86	$1.41 \times 10^{-6}$	5.85
6.00	$1.00 \times 10^{-6}$	3.40	$8.82 \times 10^{-4}$	1.93	$4.36 \times 10^{-6}$	5.36
6.50	$3.16 \times 10^{-7}$	3.48	$9.02 \times 10^{-4}$	1.98	$1.38 \times 10^{-5}$	4.86
7.00	$1.00 \times 10^{-7}$	3.52	$9.13 \times 10^{-4}$	2.00	$4.36 \times 10^{-5}$	4.36
7.50	$3.16 \times 10^{-8}$	3.52	$9.13 \times 10^{-4}$	2.00	$1.38 \times 10^{-4}$	3.86
8.00	$1.00 \times 10^{-8}$	3.52	$9.13 \times 10^{-4}$	2.00	$4.36 \times 10^{-4}$	3.36

From Fig. 3,  $1/2 \log K$  was found to be 7.35, and  $\log K = 14.70$ . The value,  $\bar{n} = 2.00$ , is a maximum for the average number of ligand molecules bound to a single metal ion.

Tetracaine HCl, 30°, 75-ml. sample,  $3.34 \times 10^{-2}$  moles/L.; Cu<sup>++</sup>,  $6.06 \times 10^{-3}$  moles/L.; NaOH,  $2.48 \times 10^{-4}$  moles/ml., 0.50-ml. increment

5.10	$7.94 \times 10^{-6}$	1.60	$3.97 \times 10^{-4}$	0.87	$1.51 \times 10^{-5}$	4.82
5.20	$6.31 \times 10^{-6}$	1.95	$4.84 \times 10^{-4}$	1.06	$1.82 \times 10^{-5}$	4.74
5.40	$3.98 \times 10^{-6}$	2.30	$5.71 \times 10^{-4}$	1.25	$2.76 \times 10^{-5}$	4.56
5.60	$2.51 \times 10^{-6}$	2.55	$6.32 \times 10^{-4}$	1.39	$4.26 \times 10^{-5}$	4.37
5.80	$1.58 \times 10^{-6}$	2.70	$6.69 \times 10^{-4}$	1.47	$6.70 \times 10^{-5}$	4.18
6.00	$1.00 \times 10^{-6}$	2.85	$7.07 \times 10^{-4}$	1.55	$1.02 \times 10^{-4}$	3.99
6.20	$6.31 \times 10^{-7}$	2.90	$7.19 \times 10^{-4}$	1.58	$1.62 \times 10^{-4}$	3.79
6.40	$3.98 \times 10^{-7}$	2.95	$7.31 \times 10^{-4}$	1.60	$2.51 \times 10^{-4}$	3.60
6.60	$2.51 \times 10^{-7}$	2.95	$7.31 \times 10^{-4}$	1.60	$3.98 \times 10^{-4}$	3.40

From Fig. 4,  $1/2 \log K$  was found to be 4.77 and  $\log K = 9.54$ .

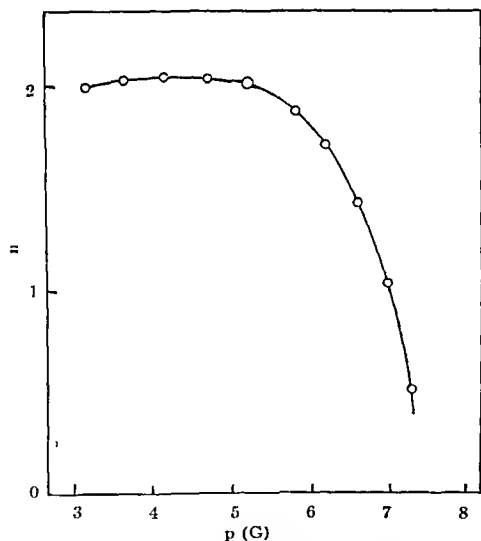


Fig. 3.—Glycine complex formation curve.

tion samples become slightly opalescent in the near neutral range, the cupric ions were probably not tightly bound to the tetracaine molecule and were being removed from solution through the formation of hydroxo complexes and hydroxides. Therefore, it is reasonable to assume that the complex formation was limited by the formation and solubility of cupric hydroxides.

Compounds having a structure similar to that of the tetracaine salt were also tested for their ability to complex with the cupric ion; it was found that the hydrochloride salts of both procaine and butethamine formed complexes with copper.

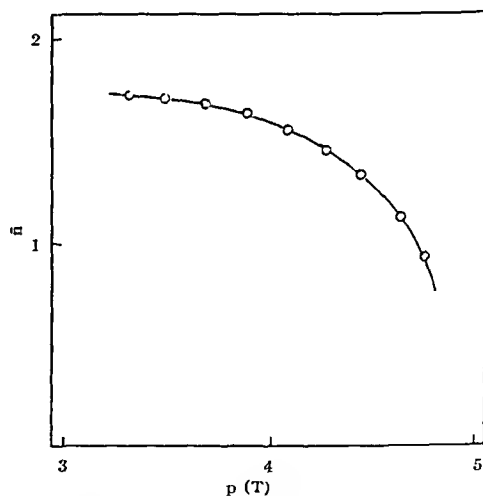


Fig. 4.—Tetracaine complex formation curve.

**Determination of Stability Constants.**—Tetracaine HCl, procaine HCl, butethamine HCl, glycine,  $\alpha$ -alanine,  $\beta$ -alanine, and serine, with and without cupric ions, were prepared and titrated as outlined. The procedure was then repeated, varying the concentrations of the metal ion.

The apparent acidity (dissociation) constants of the complexing agents, as defined by Bronsted (7), were determined and are tabulated in Table II. The concentration range of ligands varied from  $1.25$  to  $2.50 \times 10^{-3}$  moles/sample; therefore, the ionic strength of the samples was considered negligible. The literature values of the  $pK'_a$  were corrected to 30° by means of the correction factors given by Albert (10).

TABLE II.—APPARENT ACIDITY CONSTANTS OF THE COMPLEXING AGENTS<sup>a</sup>

Compound	pK'a (Found) <sup>b</sup>	pK'a (Literature) <sup>c</sup>	Reference
Glycine	9.69	9.71	(8)
$\alpha$ -Alanine	9.86	9.80	(8)
$\beta$ -Alanine	10.45	10.30	(8)
Serine	9.23	9.26	(8)
Tetracaine HCl	8.37	8.33	(9)
Tetracaine HCl <sup>e</sup>	8.59	.	.
Procaine HCl	8.99	9.05	(9)
Monocaine HCl	9.02	.	.

<sup>a</sup> Comparison between results of this investigation and literature values corrected to 30°

<sup>b</sup> At ionic strengths of approximately 0.03, except for <sup>c</sup>

<sup>c</sup> At an ionic strength of approximately 0.67

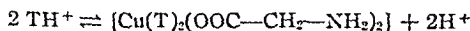
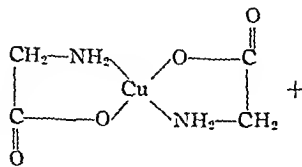
Using the method previously described, the p(B) and  $\bar{n}$  values were computed. The calculation was repeated for each concentration of cupric ion used, and the stability constant was obtained from the formation curve. Table III presents these values. The binding of the copper to the drugs and amino acids would appear to be of the type previously postulated.

**The Binding of Tetracaine HCl to Copper-Amino Acid Complexes.**—The objectives of this portion of the investigation were (a) to determine the possible direct complexation of tetracaine HCl with amino acids, and (b) to determine the possible mediation of binding by metal ions, as Klotz and Loh Ming (12) have done.

Samples of the amino acid-copper complexes, glycine,  $\alpha$ -alanine,  $\beta$ -alanine, and serine were prepared stoichiometrically in a 2:1 ratio and were titrated with sodium hydroxide in the presence of tetracaine HCl, as was previously outlined.

It was found that the amino acids did not complex directly with tetracaine HCl. The second possibility, mediation binding by the metal ion, was indicated since the pH values of the samples of the three components, tetracaine HCl + amino acid + cupric ion were lower than in any of the other solutions. Using the method for the calculation of the stability constant, as previously outlined, the stability constants and  $\bar{n}$  were calculated (considering the amino acid-copper complex as the 'metal ion'). Table IV is a tabulation of the results obtained with the four amino acids.

Ley (13) has shown that the  $\text{Cu}(\text{Glycine})_2$  complex may be altered by the addition of a foreign ligand. In this alteration the chelate rings of the original compound are opened to yield a complex. Therefore, the reaction that occurs between  $\text{Cu}(\text{Glycine})_2$  and tetracaine may be of the type:



The variation that was evident with  $\beta$ -alanine, may be due to the greater distance that exists between the amino and carboxyl groups.

TABLE III.—STABILITY CONSTANTS OF COPPER WITH VARIOUS LIGANDS<sup>a</sup>

Copper Complex	$\bar{n}$	log K Found	log K Literature <sup>b</sup> (11)
Glycine	2.00	14.7	15.0
$\alpha$ -Alanine	2.03	15.0	14.8
$\beta$ -Alanine	2.02	12.4	12.6
Serine	2.09	15.2	14.4
Tetracaine	1.71	9.6	.
Tetracaine <sup>c</sup>	1.52	9.8	.
Procaine	1.53	10.6	.
Monocaine	1.76	10.9	..

<sup>a</sup> At ionic strengths of approximately 0.03, except for <sup>b</sup> and <sup>c</sup>

<sup>b</sup> Literature value at a low ionic strength (ca 0.01)

<sup>c</sup> At an ionic strength of approximately 0.67

TABLE IV.—STABILITY CONSTANTS FOR THE BINDING OF TETRACaine HCl TO COPPER-AMINO ACID COMPLEXES

Copper-Amino Acid Complex	$\bar{n}$	log K
Copper-glycine	1.90	10.3
Copper- $\alpha$ -alanine	2.20	10.3
Copper- $\beta$ -alanine	1.30	7.7
Copper-serine	2.00	10.3

**Relationship Between pK'a and log K.**—A rough proportionality has been found to exist between the basic strength of amines and their complex forming affinity (14, 15, 16). Martell and Calvin (2) state that, in general, characteristics of the complex formation are a correlation with the basic strength of the electron donor and the effect of substitution on the donor.

Accordingly, the degree of binding (stability constant) should be directly related to the dissociation constant (pK'a) of the drug as, in fact, was found as shown in Fig 5.

It is well established that trace metals often play a part in drug mechanism. An excess of the metal ions can serve to decrease drug action substantially by binding with the key dissociating functional groups. On the other hand, an excess of the trace metals may allow the drug to elicit a greater effect at its site of action. The log K of their copper

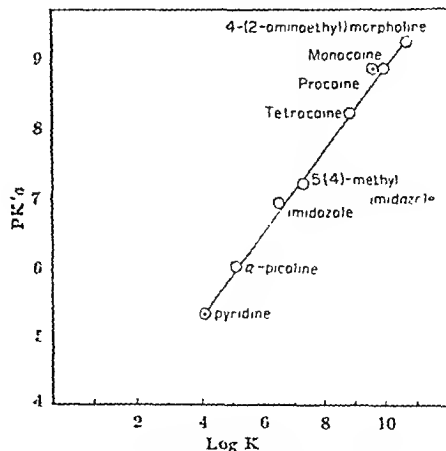


Fig. 5.—Tertiary amine complexes with copper (II) in aqueous solution.

complexes of the tertiary amines may, therefore, be related roughly to the physiological action of these agents. Accordingly, the knowledge of the physical properties and physico-chemical constants provides an avenue of approach to drug mechanism that could well lead to a more successful prediction of biological activity, both qualitative and quantitative, as well as to give an estimate of the selective toxicity of new compounds prior to pharmacological screening.

**A Biological Evaluation of the Copper-Tetracaine Complex.**—The objective of this part of the study was to determine whether or not the complexed form of tetracaine HCl affected the local anesthetic activity of tetracaine HCl. Specifically, tetracaine HCl and tetracaine HCl with varying copper concentration solutions were tested by means of corneal anesthesia and infiltration anesthesia in the guinea pig to determine any differences in the duration of action and degree of anesthesia.

The guinea pig cornea and intradermal wheal methods were selected for comparing the degree and durations of action of the solutions (17, 18, 19). Both tests are simple, rapid, and sensitive.

For the cornea anesthesia test a 1.00% tetracaine HCl solution (solution 1) was prepared. The various concentrations of cupric ion were so selected to remove, theoretically, 21% (solution 2), 39% (solution 3), and 100% (solution 4) of the tetracaine HCl.

For the wheal test, a tetracaine HCl concentration of 0.05% was chosen for comparing the effectiveness of the complexed forms. The concentrations used in the corneal method were diluted 20 times with distilled water to a 0.05% concentration. In both cases, all solutions were freshly prepared before use, and the animals and solutions were randomly selected. The investigator was unaware of the identities of the solutions until the experiments were completed. A statistical evaluation of the results is found in Table V.

In the corneal method, no significant statistical differences were noted between samples, as compared to solution 1. In the wheal method, no statistical differences were noted with solutions 2 and 3, as compared with solution 1. However, statistical significance was noted with solution 4 since this result would be had more than 95% of the time. This would indicate that the presence of cupric ions in tetracaine HCl decreased the degree of local anesthesia to a slight extent. It would appear that the presence of cupric ions immobilized the aliphatic type amine portion of

TABLE V—A STATISTICAL EVALUATION OF THE BIOLOGICAL DATA

Solution	Test	Std Deviation	Std Error of Mean	Mean Values
Corneal Method <sup>a</sup>				
1		2.83	1.00	12.63
2	0.537 > <i>p</i> 0.5	3.69	1.30	11.75
3	0.400 > <i>p</i> 0.5	3.36	1.19	13.25
4	0.391 > <i>p</i> 0.5	4.65	1.64	11.88
Wheal Method				
1		6.92	2.45	6.75
2	1.470 < 0.2 <i>p</i> > 0.1	10.72	3.78	13.38
3	1.003 < 0.4 <i>p</i> > 0.3	8.50	3.00	10.63
4	2.197 < 0.05 <i>p</i> > 0.02	14.00	4.95	18.88

<sup>a</sup> Using units of 5 min.

the local anesthetic that is thought to exert the sensory anesthesia (9). This would support the previously postulated mechanism of complexation.

An evaluation of the copper-glycine-tetracaine complex by the corneal anesthesia test showed no statistically significant differences between the complexed samples and solution 1.

## REFERENCES

- (1) Diehl, H. *Chem. Revs.*, **21**, 39 (1937).
- (2) Martell, E. A. and Calvin, M., "Chemistry of Metal Chelate Compounds," Prentice Hall, New York, N. Y., 1952, p. 78.
- (3) Albert, A., and Magrath, D., *Biochem. J.*, **41**, 534 (1947).
- (4) Bjerrum, J., "Metal Amine Formation in Aqueous Solution," P. Haase and Son, Copenhagen, Denmark, 1941.
- (5) Calvin, M., and Melchior, N. C., *J. Am. Chem. Soc.*, **70**, 3270 (1948).
- (6) Albert, A. *Biochem. J.*, **47**, 531 (1950).
- (7) Bronsted, J. N. *Chem. Revs.*, **5**, 231 (1928).
- (8) Greenberg, D. M., Ed., "Amino Acids and Proteins," C. C. Thomas, Springfield, Ill., 1951, p. 430.
- (9) Krah, M. E., Keltch, A. K., and Clowes, G. H. A., *J. Pharmacol. Exptl. Therap.*, **68**, 330 (1940).
- (10) Albert, A., *Pharmacol. Revs.*, **4**, 136 (1952).
- (11) Bjerrum, J., Schwarzenbach, G., and Sillen, L. G., "Stability Constants," Part I, Organic Ligands, The Chemical Society, London, England, 1957.
- (12) Klotz, I. M., and Loh, Ming, W. C., *J. Am. Chem. Soc.*, **76**, 805 (1954).
- (13) Ley, H., *Ber.*, **42**, 354 (1909), through Bailar, J. C., "The Chemistry of Coordination Compounds," Reinhold Publishing Corp., New York, N. Y., 1956, p. 37.
- (14) Carlson, G. A., McReynolds, J. P., and Verhock, F. H., *J. Am. Chem. Soc.*, **67**, 1334 (1945).
- (15) Bruehlman, R. J., and Verhock, F. H., *ibid.*, **70**, 1401 (1948).
- (16) Calvin, M., and Wilson, K. W., *ibid.*, **67**, 2003 (1945).
- (17) Beyer, K. H., Latven, A. R., Freyburger, W. A., and Parker, M. P., *J. Pharmacol. Exptl. Therap.*, **93**, 388 (1948).
- (18) Chance, M. R. A., and Lobstein, H., *ibid.*, **82**, 203 (1944).
- (19) Sinha, H. B., *ibid.*, **57**, 199 (1936).

# The Application of $\Delta\epsilon$ -Analysis to Pharmaceuticals: The Determination of Eugenol\*

By JULIUS C. DEMETRIUS, Jr.†, and JOSEPH E. SINSHEIMER

The  $\Delta\epsilon$ -method of analysis, which was developed for the investigation of lignins, involves the selective modification of a given chromophore in a mixture of chromophores. This method permits the quantitative determination of a single ultra-violet-absorbing compound in mixtures of such absorbing materials. The analyses of eugenol in clove oil, in eugenyl acetate, and in two formulations are described as illustrations of the pharmaceutical application of this technique. The absorbance at 296  $m\mu$  of an aliquot of the eugenol-containing preparation was determined in basic solution. The change in absorbance was then compared to the bathochromic displacement of a standard eugenol solution with a corresponding change in pH.

THE STUDY of the phenolic compounds of various lignins by Aulin-Erdtman (1, 2) and by Goldschmid (3, 4) was based in part upon the development of " $\Delta\epsilon$ -curves." In these investigations the  $\Delta\epsilon$ -curves were obtained by subtracting the absorbances of the U. V. spectra of lignin derivatives in solution of low pH from their corresponding spectra in solution of high pH. By comparing  $\Delta\epsilon$ -curves obtained in this manner to those obtained from model compounds in a similar manner, both qualitative and quantitative studies of phenolic compounds, even in the presence of other U. V. absorbing material, were possible.

The method need not be limited to the bathochromic displacement of phenols in alkaline solution. Aulin-Erdtman (5) in a review article points out that the  $\Delta\epsilon$ -method may be applied whenever the absorption properties of a given chromophore can be modified selectively in the presence of a mixture of chromophores. A  $\Delta\epsilon$ -curve is still obtained by subtracting the spectrum of the starting material from the spectrum of the product. The absorbance of any non-modified chromophores is thereby cancelled out. Thus, in those cases where selective modification of the chromophore can be accomplished,  $\Delta\epsilon$ -analysis has the advantage of the analysis of a single absorbing material in a mixture of absorbing materials without the necessity for prior separation.

This technique should have wide application to pharmaceutical determinations. It is the purpose of this investigation to apply  $\Delta\epsilon$ -analysis to a quantitative pharmaceutical problem. The lignin studies involved eugenol as one of the model compounds. A direct pharmaceutical extension of these studies was undertaken in the assay of clove oil for its eugenol content and the

assay of eugenol in a pharmaceutical product. In addition, the procedure developed for the assay of clove oil was tested by its application to a sample of eugenyl acetate.

## EXPERIMENTAL

Ultraviolet absorption spectra and quantitative measurements were made with a Beckman, model DU spectrophotometer. All quantitative measurements were the average of six determinations. After the first three determinations, the cells containing the sample solution and blank solution were interchanged.

**Materials.**—Eugenol (Eastman Kodak Co.) was fractionated by vacuum distillation through a helices-packed column. The refractive index of the purified sample was  $n_D^{20} = 1.5410$ . Eugenyl acetate (Aldrich Chemical Co.) was fractionated by a similar distillation and crystallized from alcohol-water. The melting point of the purified sample was 30–31°. Clove oil U. S. P. (Magnus, Mabec and Reynard, Inc.) had a total phenolic content of 87% as determined by the U. S. P. XV assay. All other materials were of C. P. grade and were used without further purification.

**Determination of Eugenol Spectra.**—An accurately weighed sample of 59 mg. of eugenol was diluted with 50% alcohol-water to 250 ml. in a volumetric flask. A series of 10-ml. portions of this solution was transferred to 100-ml. volumetric flasks and diluted to the mark with a series of buffer solutions prepared from standard solutions of sodium hydroxide and sulfuric acid. The pH of each of these eugenol solutions was measured by a Beckman Zeromatic pH meter and its U. V. spectrum determined. In this manner eleven spectra of eugenol in solutions ranging from pH 2.5 to 13.5 were obtained.

**The Determination of  $\Delta\epsilon$  for Eugenol.**—Samples of about 50 mg. of eugenol were accurately weighed and diluted with alcohol in a 250-ml. volumetric flask. One 10-ml. portion of this solution was diluted with water and 1 ml. of 1.000 *N* sodium hydroxide in a 100-ml. volumetric flask. A second 10-ml. aliquot of the alcoholic eugenol solution was transferred to a 100-ml. volumetric flask and diluted with water and 1 ml. of 0.100 *N* sulfuric acid. The absorbance of the alkaline solution was determined relative to the acid solution in the ref-

\* Received August 21, 1959, from the College of Pharmacy, University of Rhode Island, Kingston.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: Mead Johnson and Co., Evansville, Ind.



erence cell at 296  $m\mu$ . Blank solutions were also prepared, and any relative absorbance was determined in a like manner in order to correct for any contribution due to solvents and reagents. Seven such determinations were made. The alkaline solutions were recorded with a pH of  $12.0 \pm 0.2$  and the acid solutions with a pH of  $3.0 \pm 0.2$ .

**The Determination of Eugenol in Clove Oil and in Eugenyl Acetate.**—Samples of about 60 mg. were accurately weighed and placed together with 3 ml. of 1 *N* sodium hydroxide and 15 ml. of alcohol in a 250-ml. volumetric flask. After the mixture was shaken for five minutes at room temperature, it was heated in a boiling water bath for fifteen minutes. During this time the flasks were shaken at five-minute intervals. The flasks were cooled to room temperature and alcohol was added to 250 ml. At the same time a control solution without sample was also prepared in this manner. Ten-milliliter aliquots of the saponified samples and the control were then diluted and buffered; the relative absorbances at 296  $m\mu$  were determined essentially as described under the determination of  $\Delta\epsilon$  for eugenol. The only modification required was the addition of 2 ml. of 0.100 *N* sulfuric acid to obtain the desired pH of  $3.0 \pm 0.2$  for the reference solution.

**The Determination of Eugenol in Formulations.**—An aliquot of the formulation was chosen to obtain a concentration of about 2 mg. per 100 ml. of eugenol in the reference and alkaline solutions. The procedure described under  $\Delta\epsilon$  for eugenol was then followed.

**Calculations and Definitions.**— $\Delta\epsilon$  for eugenol:  
 $\Delta\epsilon = (\Delta A \times 164.2)/c$

Percentage of eugenol:  $\% = (\Delta A \times 164.2 \times 100)/(c \times \Delta\epsilon)$

Concentration:  $c = (\Delta A \times 164.2)/\Delta\epsilon$

Where:  $\Delta\epsilon$  = absorbance at 296  $m\mu$  of a 1*M* solution of eugenol in base less the absorbance of a 1*M* solution in acid.  $\Delta A$  = the observed absorbance at 296  $m\mu$  of a given concentration of eugenol in basic solution less the absorbance of same concentration of eugenol in acid solution.  $c$  = concentration in Gm./L.

## RESULTS AND DISCUSSION

Figure 1 represents the spectra of eugenol in (a) acid to neutral solution and (b) in alkaline solution. As is typical of phenols, there is a pronounced bathochromic displacement of both the E and B bands and an increase in the intensity of absorption of these bands. When the acid spectra is subtracted from that of the alkaline spectra, the  $\Delta\epsilon$ -curve illustrated in Fig. 2 is obtained.

The same  $\Delta\epsilon$ -curve is produced even in the presence of other U. V.-absorbing compounds provided that these compounds show no response to changes in pH.  $\Delta\epsilon$ -curves can be used in a manner analogous to normal U. V. curves for qualitative and quantitative purposes. Thus, the maxima at 246 and 296  $m\mu$  should serve as the basis for quantitative measurements.

It appears that the 246  $m\mu$  maximum, because of its greater intensity, would be the most useful for quantitative measurements. However, in actual

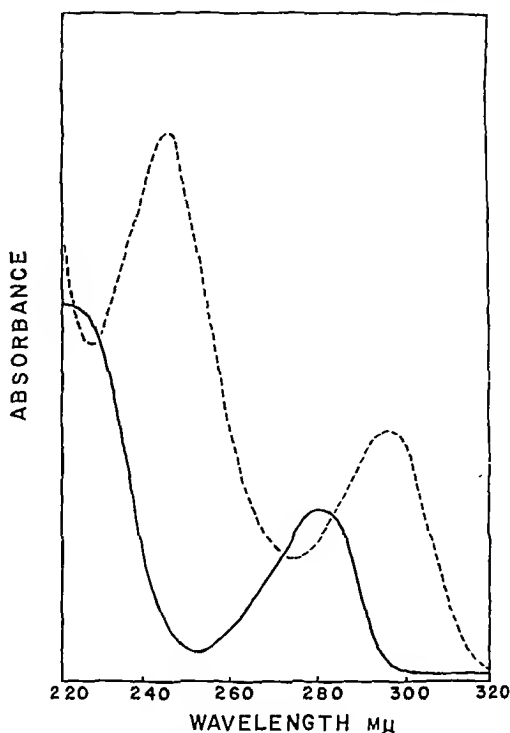


Fig. 1.—Ultraviolet absorbance spectra of eugenol; — pH 7.0; ---- pH 12.3.

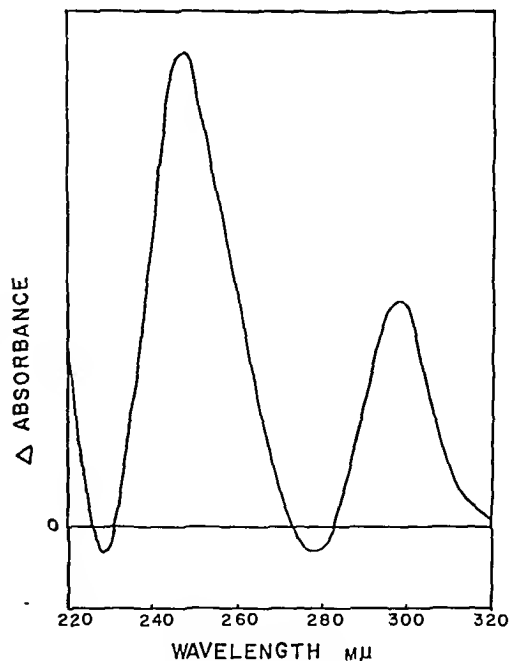


Fig. 2.— $\Delta\epsilon$  ultraviolet absorbance spectrum of eugenol at pH 12.3 with the eugenol spectrum at pH 7.0 as a reference.

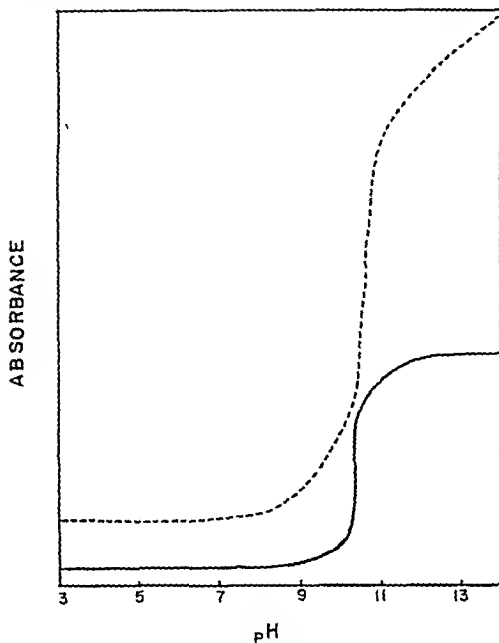


Fig. 3.—Maximum absorbance of eugenol as a function of pH; — maximum at 296  $m\mu$ ; --- maximum at 246  $m\mu$ .

practice this is not the case, since the 296  $m\mu$  maxima showed greater stability with changes in alkaline pH than did the 246  $m\mu$  maxima and, therefore, would require a less critical control of pH for quantitative comparisons. The change in absorbance of these peaks as a function of change in pH is illustrated in Fig. 3.

To serve as a basis for the quantitative comparison of eugenol solutions, the  $\Delta\epsilon$  for the 296  $m\mu$  maxima was determined from standard solutions. The average of seven determinations gave a value of 3,886 for a molar solution of eugenol of a pH of  $12.0 \pm 0.2$  over that of a molar solution of a pH of  $3.0 \pm 0.2$  at 296  $m\mu$ .

Table I is a summary of the results of the determination of the percentage of eugenol in a sample of clove oil based upon this value for  $\Delta\epsilon$ . As a partial test of the procedure there is also included in Table I the results of the analysis of a sample of eugenyl acetate.

In order to investigate the application of  $\Delta\epsilon$ -analysis to a pharmaceutical formulation the following mouth wash, formula A, was compounded.

Saccharin, soluble...	0.025 Gm.
Fuchsin, basic...	0.005 Gm.
Peppermint oil...	0.100 Gm.
Eugenol...	0.100 Gm.
Alcohol.....	75.00 cc.
Distilled water to	250.00 cc.

Table II presents the results of the analysis of this solution for its eugenol content.

TABLE I.—ANALYSIS OF EUGENOL IN CLOVE OIL AND EUGENYL ACETATE

Sample	Clove Oil, %	Eugenyl Acetate, % (79.6% Calcd.)
1	85.4	80.0
2	83.8	79.9
3	85.7	81.2
4	86.7	79.5
5	86.0	...
6	84.9	...
7	85.8	...
Av.	85.5	80.2

TABLE II — ANALYSIS OF EUGENOL IN MOUTH WASH FORMULAS

	Formula A, Gm.	Formula B, Gm.
Amount present	0.1004	0.1124
Found 1	0.0980	0.1090
2	0.0980	0.1107
3	0.0997	0.1104
4	0.0976	0.1101
5	0.1001	0.1094
6	0.0972	0.1103
Av.	0.0984	0.1099

The above formula was also modified by reducing the peppermint oil to 0.06 Gm. and including 0.06 Gm. of cassia oil which possesses U. V. absorption of high intensity. The results for the analysis of eugenol in the modified formula, formula B, are also presented in Table II.

It should be noted that the U. V. spectrum of cassia oil shows a limited sensitivity to changes in pH, and results recorded in Table II would have been about 10% lower unless corrected for this change. That is, the analysis of formula B required that the absorbance of a control solution without eugenol also be determined and be applied as a correction to the analysis of the complete formula. Formula B, therefore, demonstrates a limitation of  $\Delta\epsilon$ -analysis: the sensitivity of the other compounds in a mixture to the conditions used to modify the chromophore of interest. This limitation can be overcome in the analysis of dosage forms by running a control as noted in the analysis of formula B or by applying a simultaneous equation determination to the  $\Delta\epsilon$ -curve. If such corrections cannot be applied as, for example, in the assay of some natural products, the extent of these errors can readily be determined and, to a great degree, corrected by comparing the complete  $\Delta\epsilon$ -spectrum of mixture to that of the standard compound.

#### References

- (1) Aulin-Erdtman, G., *Scensk Papperstidn.*, 55, 745 (1952); *Chem. Abstr.*, 48, 11780a (1954).
- (2) Aulin-Erdtman, G., *ibid.*, 56, 287 (1953); *Chem. Abstr.*, 47, 10542i (1953).
- (3) Goldschmid, O., *Anal. Chem.*, 26, 1421 (1954).
- (4) Maranville, L. F., and Goldschmid, O., *ibid.*, 26, 1423 (1954).
- (5) Aulin-Erdtman, G., *Chem. & Ind.*, 74, 581 (1955).

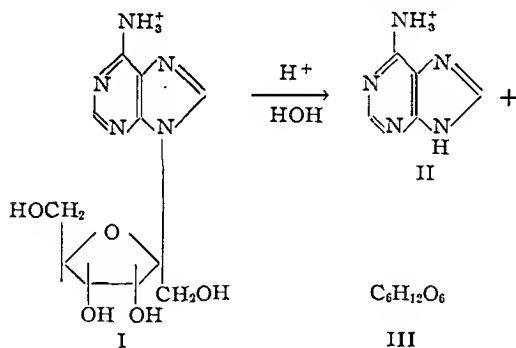
## Psicofuranine: Correlation of Assay Methods in Acid Degradation Studies\*

By EDWARD R. GARRETT and LADISLAV J. HANKA

The unnatural nucleoside, psicofuranine, is bimolecularly hydrolyzed by acid to adenine and the sugar psicose. Adenine appears to reverse the biological activity of psicofuranine in the plate-disk method against *S. aureus* so that standard curves must be prepared with the same amount of adenine as the material to be assayed. When this phenomenon is accounted for, the chemical and biological assays are shown to correlate. The kinetic constants for the acid catalyzed hydrolysis of the nucleoside have been determined.

THE NEW ANTIBIOTIC, psicofuranine (1), has been assigned the structure 6-amino-9-D-psicofuranosylpurine by Schroeder and Hoeksema (2). This structure has also been proposed for angustmycin C by Hsu (3). Properties and assay methods have been studied (4-14).

The purpose of this paper is to consider the acid catalyzed degradation of psicofuranine, I, by hydrolysis to adenine, II, and psicose, III;



as measured by both biological (6, 12) and chemical (10, 11) assays, and to determine the significance of the correlation between the assay methods.

## EXPERIMENTAL

**Kinetics of the *In Vitro* Degradation of Psico-furanine by Acid at 40°.**—Sufficient psicofuranine, generally 25 mg., previously dried at 60° under high vacuum for forty-eight hours, was weighed into a tared 25-ml. volumetric flask, and made up to volume with the solvent of the appropriate hydrochloric acid concentration (see Table I). This sol-

TABLE I.—RATE CONSTANTS FOR THE ACID HYDROLYSIS OF 1 MG./ML.<sup>a</sup> PSICOFURANINE AT 40° AS A FUNCTION OF HYDROCHLORIC ACID CONCENTRATION

Runs	$10^4 k$ (sec. <sup>-1</sup> )	[HCl] <sub>A</sub>	pH	Temp., ° C.
1	0.538	0.010	2.13	40.0
2	1.58	0.025	1.65	40.2
3	3.89	0.050	1.36	40.2
4	6.20	0.075	1.20	40.2
5	9.32	0.100	1.06	40.2
6 <sup>a</sup>	9.14	0.100	1.05	39.3
7 <sup>a</sup>	8.98	0.100	1.06	39.5
8 <sup>c</sup>	8.21	0.100	1.05	39.2
9 <sup>d</sup>	9.17	0.100	1.05	39.4
10 <sup>d</sup>	9.17	0.100	1.05	39.4

<sup>a</sup> Except for run 6 at 0.42 mg./ml. and run 7 at 0.61 mg./ml.

<sup>b</sup> Accurate within a run to  $\pm 0.05^\circ$ .

<sup>c</sup> Correlated with bioactivity; no apparent reason for low estimate of rate.

<sup>d</sup> Correlated with bioactivity and run simultaneously except that an initially equimolar amount of adenine was added to run 10.

vent had been previously equilibrated in the 40° constant temperature bath. The resultant solutions were placed in the 40° temperature bath and at recorded time intervals assayed by the procedure of Forist (11) which has been applied to the chemical determination of psicofurarine in blood plasma and serum (10). Prior to the assay a 1-ml. aliquot was pipetted into a 5-ml. volumetric flask containing sufficient NaOH to slightly over-neutralize the aliquot so as to halt the acid degradation. This solution was then diluted with water up to volume and the assay procedure applied.

The rate of acid degradation of psicofuranine should be bimolecular, proportional to the psicofuranine molarity,  $[P]$ , and the molarity of the hydrochloric acid. The apparent molarity,  $[HCl]_A$ , should be corrected for the amount of psicofuranine of  $pK_a' 3.9$ , determined by the pH at half-neutralization, which provides a true molarity of  $[HCl]_A - [P]_0 = [HCl]$  where  $[P]_0$  is the initial concentration of the antibiotic. The  $pK_a'$  of the hydrolysis product adenine is almost identical so that the acid concentration will not vary throughout the reaction. Thus

$$-d[P]/dt = k_H^+ \{ [HCl]_A - [P]_0 \} [P] \quad (\text{Eq. 1})$$

Equation 1 may be integrated as a pseudo first-order equation so that

$$\log[P] = -kt/2.303 + \log[P]_0 \quad (\text{Eq. 2})$$

where

$$k = k_H^+ \{ [\text{HCl}]_A - [\text{P}]_0 \} \quad (\text{Eq. 3})$$

Typical plots according to Eq. 2 are given in Fig. 1 where the pseudo first-order nature of the acid degradation is confirmed by the linearity of the plots. The logarithm of the assayed absorbance which is proportional to the psicofuranine concentra-

\* Received November 23, 1959, from the Research Laboratories of The Upjohn Co., Kalamazoo, Mich.

Grateful acknowledgments are given to (Mrs.) Lillian G. Snyder for excellent technical assistance, to Mr. Ernest Markovich for the operation of the digital computer in statistical correlations, and to Dr. Arlington A. Forist for helpful advice and discussions on the use of the chemical assay.

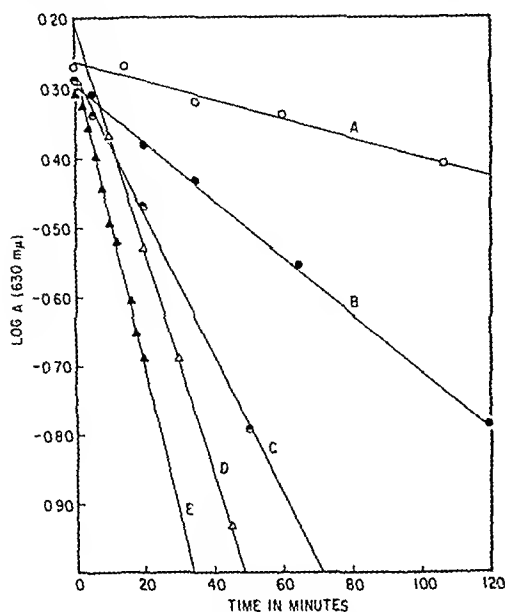


Fig 1—The pseudo first-order plots of psicofuranine degradations at 40° in varying molarities of HCl at 40.0°. The curves, HCl molarity and apparent pH are: A, 0.01 M, 2.13; B, 0.025 M, 1.65; C, 0.050 M, 1.36; D, 0.075 M, 1.20; E, 0.10 M, 1.05. The absorbance values,  $A$ , at 630 mμ, are proportional to psicofuranine content and are the readings determined by the assay procedure of Forst (11).

tion is plotted against time. The catalytic effect of acid concentration is apparent from the increase in negative slope with the increase in hydrochloric acid molarity. The evaluated rate constants for the pertinent conditions used are given in Table I.

If the reaction is truly bimolecular with respect to acid and psicofuranine, the apparent first-order rate constant at constant hydrochloric acid concentration should not vary significantly with psicofuranine concentration. This point is confirmed by the consistency of the apparent first-order rate constants at 0.100 M HCl for three different initial concentrations of psicofuranine (see runs 5 through 10 in Table I). The dependence of the apparent first-order rate constants,  $k$ , on HCl concentration is graphically given in Fig 2 according to Eq 3. The apparent  $[HCl]_A$  value when  $k = 0$  is 0.004 which agrees well with the psicofuranine concentration at 1 mg/ml, i.e.,  $[P]_0 = 0.0037$ . The slope of Fig 2 is  $k_H^+ = 0.00374$  moles/L/min. The per cent purity of the psicofuranine by titration on assumption of the molecular formula (2) is 96.3%.

**Correlation of the Chemical and Biological Assays of Psicofuranine.**—In an attempt to correlate the chemical (10, 11) and biological (6) assays of psicofuranine, a kinetic study was made at 40° in 0.100 M HCl and aliquots removed and assayed by both methods. The aliquots for biological assay were immediately neutralized and submitted on the basis of 80 γ/ml of the original material. The same psicofuranine as used in the study in the same slightly saline solutions was used to establish the standard curve for the plate disk assay, i.e., zone diameter in mm vs log concentration. A discrep-

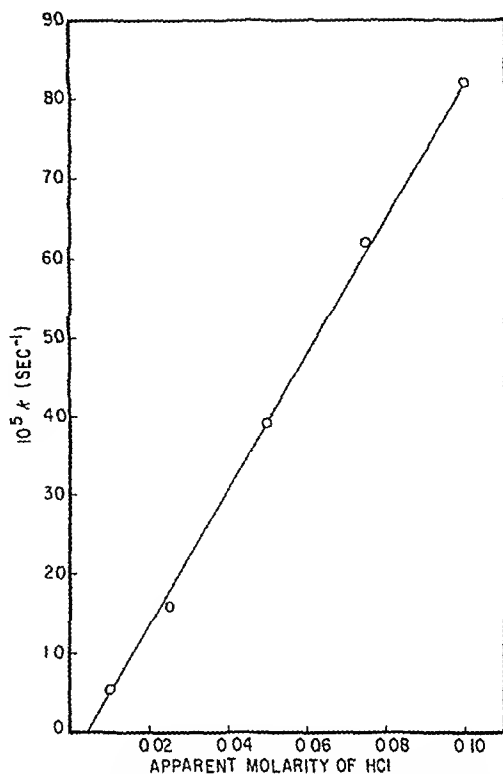


Fig 2—Rate constants,  $k$  in sec<sup>-1</sup>, for the pseudo first order degradation of psicofuranine as a function of apparent HCl molarity at 40°.

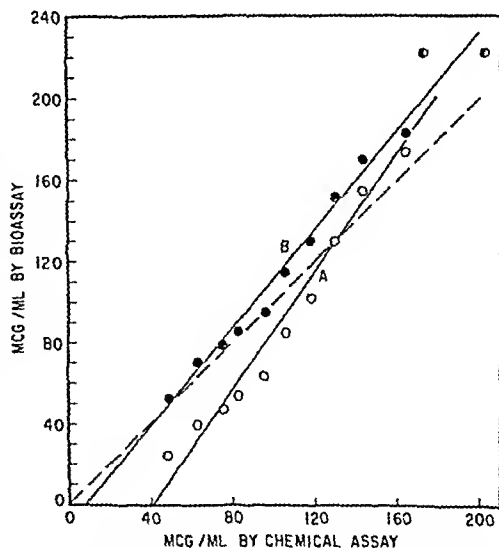


Fig 3—Correlation of disk-plate assay against *S. aureus* and the spectrophotometric measurement on the product from reaction of degraded (0.1 N HCl, 40°) psicofuranine with diphenylamine. Curve A is based on the standard curve for bioassay set up on psicofuranine alone. Curve B is based on the standard curve for bioassay predicted for psicofuranine with the amounts of adenine present in the aliquots to be assayed. The dashed line is theoretical.

any became immediately apparent. A plot of the potency by biological assay against purity by chemical assay showed an intercept not passing through the origin (see Fig. 3, curve A). Either this was an artifact of one of the assays or a certain fraction of the intact nucleoside had no biological activity.

This latter hypothesis and that of an artifact in the chemical assay appeared improbable since the acid degradations are elegantly pseudo first-order (see Fig. 1). Also, the chemical assay absorbance was experimentally shown to be a linear function of the concentration of the nucleoside.

A parallel experiment was again run (runs 9 and 10, Table I) whereby aliquots of the acid degrading psicofuranine were again removed, neutralized, and assayed by both procedures. One of these studies was as before. An initially equimolar amount of adenine, a hydrolytic product of the acid catalyzed psicofuranine degradations, was added to the other.

The standard curves for the psicofuranine plate-disk assay using *S. aureus* are given for these studies in Fig. 4 where curve A is the curve for psicofuranine alone and curve B is for psicofuranine with equimolar amounts of adenine

The statistics for the regressions of bioassay on chemical assay are given in Table II. If the variation about regression is completely assigned to the bioassay, the estimated standard deviation of a single assay,  $S_y$ , is  $\pm 10$   $\gamma$ /ml for material assayed in the range 40–80  $\gamma$ /ml, i. e., an estimated error of 12% in bioassay.

## DISCUSSION

**The Apparent Discrepancy Between the Chemical and Biological Assays.**—The bioassay of aliquots of a psicofuranine-0.1 *M* HCl solution maintained at 40° gave significantly less estimates of potency with time than the chemical assay which measured intact nucleoside (see curve A, Fig. 3). However, when adenine, a product of the hydrolysis, was added to the psicofuranine, a diminution of zone size for a comparable psicofuranine concentration was apparent in the standard curves for the plate-disk assay. Compare curves A and B in Fig. 4. If the point on the psicofuranine standard curve (curve A, Fig. 4) of 80 mcg./ml. is assumed as valid for no adenine present in the assay of psicofuranine and if the point on the equimolar psicofuranine-adenine standard curve (curve B, Fig. 4) of 40 mcg./ml. is as assumed as valid for the half-hydrolyzed psicofuranine, then curve C, Fig. 4, can be constructed which could be the standard curve for assay when the levels of adenine in the acid-degraded nucleoside are considered. This assumes, of course, logarithmic effect on the diminution of zone size by the addition of adenine. A complete exposition of effects will be published by Hanka (12).

When the constructed standard curve C, Fig. 4, is used rather than the standard curve A, Fig. 4, without adenine, the regression of bioassay on chemical assay becomes curve B, Fig. 3, and as per the statistics of regression, Table II, this regression passes through the origin within experimental error. The chemical assay can be concluded as a true measure of biologically active psicofuranine.

Confirmatory evidence was obtained from the

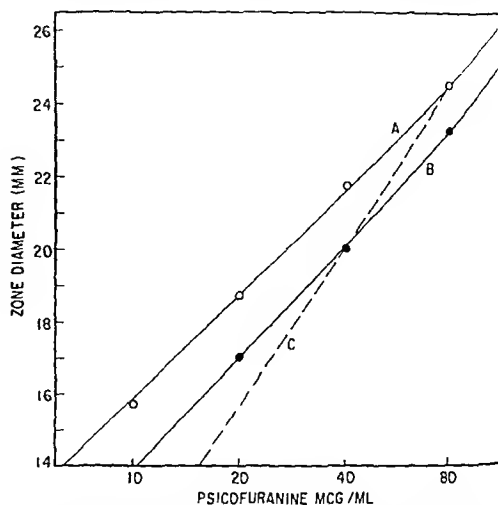


Fig. 4.—Standard curves for the disk-plate assay of psicofuranine using *S. aureus*, the zone size in mm. against the concentration in  $\gamma$ /ml. Curve A is for psicofuranine alone, curve B is for psicofuranine with a molar equivalent of adenine, the curve C is the constructed curve to account for the effect of adenine on zone size for a given antibiotic concentration when adenine is produced during acid catalyzed degradation.

TABLE II.—STATISTICS OF THE REGRESSION OF PSICOFURANINE CONTENT BY THE BIOLOGICAL ASSAY ( $y$ ) AGAINST THE CHEMICAL ASSAY ( $x$ )<sup>a</sup>

	Biological Assay Based on Standard Curves	
	Psicofuranine Alone	Psicofuranine Corrected for Adenine Effects
Equation of regression, $y = mx + b$	$y = 1.46x - 61.2$	$y = 1.22x - 11.2$
Standard deviation of slope, $S_m$	0.090	0.066
Standard deviation of intercept, $S_b$	11.4	8.3
$tS_m$	0.202	0.15
$tS_b$	25.3	18.5
Standard deviation about regression, $S_y$	14.6	10.3
Number of items, $n$	12	12

<sup>a</sup> The psicofuranine aliquots were taken at various times on degradation in 0.100 *M* HCl at 40°.

paper chromatography of the acid-degraded psicofuranine at the point in time when the biological assay (based on an adenine-free standard curve) was zero and the chemical assay was ca. 30% of the original nucleoside content. Definite amounts of nonhydrolyzed psicofuranine in the magnitude expected were separated by this chromatography and coincided in position and color development with the psicofuranine controls (5).

## SUMMARY

The kinetic investigations of the acid catalyzed degradations of psicofuranine prove that the hydrolysis is bimolecular in acid and nucleoside.

Correlation of bioassay and chemical assay for psicofuranine produced an estimated standard deviation in per cent of assay value of 12 per cent for the former which must be controlled for adenine content. The chemical assay is a valid measure of psicofuranine. Adenine reverses the biological activity of psicofuranine as assayed by the plate-disk method.

## REFERENCES

(1) Eble, T. E., Hoeksema, H., Boyack, G. A., and Savage, G. M., *Antibiotics & Chemotherapy*, 9, 419(1959)

- (2) Schroeder, W., and Hoeksema, H., *J. Am. Chem. Soc.*, 81, 1767(1959).  
 (3) Hsu, Y., . . . . . (1958)  
 (4) Vavra, J., . . . . . iminoff, P.,  
 and Koepsell, H., . . . . . 427(1959).  
 (5) Sokolski, . . . . . F. E., *ibid*,  
 9, 436(1959).  
 (6) Hanka, L. J., Burch, M. R., and Sokolski, W. T.,  
*ibid*, 9, 432(1959)  
 (7) Lewis, C., Reames, H. R., and Rhuland, L. E., *ibid*,  
 9, 421(1959)  
 (8) Evans, J. S., and Gray, J. E., *ibid*, 9, 675(1959).  
 (9) Wallach, D. P., and Thomas, R. C., *ibid*, 9, 722  
 (1959)  
 (10) Forist, A. A., Theal, S., and Hoeksema, H., *ibid*, 9,  
 685(1959)  
 (11) Forist, A. A., *Anal. Chem.*, 31, 1767(1959)  
 (12) Hanka, L. J., *J. Bacteriol.*, in press  
 (13) Garrett, E. R., *J. Am. Chem. Soc.*, 82, 827(1960)  
 (14) Garrett, E. R., Thomas, R. C., Wallach, D. P., and  
 Alway, C. D., *J. Pharmacol. Exptl. Therop.*, in press

# A Pharmacologic Study of the Effects of Various Pharmaceutical Vehicles on the Action of Orally Administered Phenobarbital\*

By MARVIN H. MALONE†, ROBERT D. GIBSON, and TOM S. MIYA‡

The  $ED_{50}$ - $ED_{95}$ / $LD_5$  of phenobarbital sodium orally to rats 5 cc./Kg. was 126-190/240 mg./Kg. Constant dosage for this study was 200 mg./Kg. or equivalent. Normalcy, instead of righting reflex regained, was used to define termination of narcosis. Phenobarbital sodium was more efficient than phenobarbital when given in aqueous agar solution-suspensions. Increasing viscosity by increasing sucrose concentration of vehicle progressively lengthened induction time. There was more rapid absorption from hypotonic than hypertonic solutions of phenobarbital sodium; yet there was less rapid absorption when given 10 cc./Kg. than 5 cc./Kg. using an 80 per cent sucrose vehicle. A sex and species variation in response was noted. Calculations utilizing log transformation data were used to summarize most accurately results obtained.

THE PRESENT CONCEPT of an elixir is a clear, sweetened hydroalcoholic liquid intended for oral use which may or may not contain active medicinal agents. Considerable attention has been given to elixirs as vehicles for drugs but most of this research has been purely pharmaceutical in nature, and has had as its goal increased pharmaceutical elegance rather than increased therapeutic efficiency. However, Seeborg and Dille (1) have studied the comparative gastrointestinal absorption of barbital, barbital sodium, and barbital elixir in the cat. Blood levels of barbital were reported one-half hour

after oral dosing, but no data as to comparative pharmacologic response were presented. Hazleton and Helleman (2) have conducted a thorough investigation of the effects of sucrose, dextrose, glycerol, propylene glycol, and ethanol on the response of sodium pentobarbital and Metrazol administered orally to mice. Their work indicated that the pharmacodynamics of an elixir would be characteristic only for that specific elixir and dependent primarily upon the pharmacologic nature of the medicinal agent and its concentration, and secondarily, upon the physical and pharmacologic nature of the specific ingredients that constitute the elixir vehicle and their relative concentrations. Their results were obtained on mice not fasted prior to testing, and consecutive testing was practiced with a minimum period of only one week between experiments.

The majority of research on barbiturates has been done with those classified as ultrashort to

\* Received August 21, 1959, from the Department of Pharmacology, College of Pharmacy, University of Nebraska, Lincoln.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

Abstracted in *Federation Proc.*, 18, 418(1959).

The major portion of this article is taken from the Doctoral Thesis of Marvin H. Malone.

† Fellow of the American Foundation for Pharmaceutical Education, 1956-1958. Present address: College of Pharmacy, University of New Mexico, Albuquerque.

‡ Present address: Department of Pharmacology, Purdue University, Lafayette, Ind.

intermediate in duration of action. Phenobarbital, a long-acting barbiturate, is the most widely used barbiturate in this country and has a wide variety of uses. Most pharmacologic research has concerned itself with the effects of barbiturates after their parenteral administration, in spite of the fact that most are consumed orally. This investigation was undertaken to elucidate the pharmacodynamics of phenobarbital elixir U. S. P. XIV (3).

## EXPERIMENTAL

**General.**—Nebraska stock white rats were raised in the animal quarters of this laboratory from Wistar strain rats. After sex-separation at the age of four to six weeks, they were permanently numbered and housed in large colony cages. Animal quarters were kept at a temperature of approximately 25°. Diet consisted of Purina laboratory chow checkers and water, both *ad libitum*. White rats termed Manor Farm stock are animals of the Wistar strain purchased from Manor Farms, Staatsburg, New York, and maintained for one month prior to test in the animal quarters of this laboratory under conditions identical to those described for Nebraska stock rats. All rats were tested at approximately thirty weeks of age. White mice used were purchased from J. C. Landis Co. of Hagerstown, Maryland, and were of Webster-Swiss stock. They were housed for one week prior to test in the animal quarters of this laboratory under conditions as described for Nebraska stock rats. Only mice within the weight range of 16–22 Gm. were used for experimentation.

Twenty-four hours prior to test, all food was removed from the cages and the cages were swept clear of food particles and feces. Both rats and mice were housed in cages with coarse wire screen floors that allowed passage of feces. During fasting, water was allowed *ad libitum*. During the test period each animal was kept in an individual container and water was withheld. Laboratory temperature was maintained at 25.5–27.8° during the tests, as animals anesthetized with phenobarbital are markedly poikilothermic. All animals with loss of righting reflex were turned every hour to prevent hypostatic pneumonia. Prior to dosing, all animals were randomized and weighed to the nearest gram. Technique of oral administration was that described by Holck, *et al.* (4), utilizing a Davol No. 8 feeding tube attached to a precision grade syringe. The length of feeding tube allowed to enter the rat from the apparatus was 120 mm. This length just passed into the cardiac portion of the stomach of male and female rats of this age and weight. Oral dosing of mice was accomplished with a precision grade syringe attached to a 20-gauge, one and one-half-inch needle, the point of which had been cut off, edges rounded inward, and polished. Dosage volume was 5 cc./Kg. for rats and 20 cc./Kg. for mice, unless otherwise stated.

Positive loss of righting reflex (LRR) was recorded when the animal lay quietly on its side and did not attempt to right itself after four consecutive hand turnings of the animal. Positive recovery of

righting reflex (RRR) was noted when the animal maintained itself in an upright position and promptly resisted four consecutive attempts to place the animal first on one side and then on the other. Time of normalcy (N) was noted when four consecutive attempts by the investigator failed to place first the right and then the left hind leg of the rat in an extended position back of the animal. Furthermore, normalcy was not recorded unless the rat was grossly free of ataxia when placed on the floor free to roam. Time of death was recorded upon the cessation of respiration. After death all animals were autopsied. Having once been on test, the surviving rats and mice were not used for further experimentation. All weighings of drugs were done using an analytical balance. Solution-suspensions were prepared in volumetric flasks of at least 50-cc. capacity at 20°, and were administered within twenty-four hours after preparation.

## RESULTS

**Phenobarbital Sodium Dose-Response Curve.**—Documentation of the oral dose-response curve of phenobarbital sodium was necessary as a point of departure for the following work. Results are summarized in Table I. Pooling the male and female data the oral narcotic ED<sub>50</sub> as calculated by the log probit method (5) was 126 mg./Kg. with a standard error of  $\pm 11.5$ . Assuming a parallel lethal slope, the oral LD<sub>50</sub> was estimated to be 360 mg./Kg. The oral therapeutic index (LD<sub>50</sub>/ED<sub>50</sub>) and safety index (LD<sub>5</sub>/ED<sub>95</sub>) were estimated to be 2.86 and 1.26, respectively. The 3.36% solution used to dose the 168.18 mg./Kg. dosage level was about isotonic. The slower induction time seen with the next higher dosage group could indicate less rapid absorption by the hypertonic 4% solution, in spite of the increase in dosage.

The common end point used to indicate termination of narcosis is RRR. However, for long-acting barbiturates such as phenobarbital where detoxification rates are slow and excretion rates equally so, this end point was not adequate. Animals receiving the higher doses of phenobarbital sodium were observed to have as many as five positive RRR end points. The borderline between sleep and nonsleep was so narrow that the animals were temporarily aroused by minor laboratory noises such as conversation, only to return to sleep once the disturbance had ceased. Once N was achieved, the rats did not relapse. Since the ED<sub>95</sub> was calculated to be 190 mg./Kg., the constant equimolecular dosage adopted for all following work was 200 mg./Kg. for phenobarbital sodium and 182.7 mg./Kg. for phenobarbital. At this dosage level any potentiation or antagonism of the barbiturate response should be apparent.

**Toxicity of Phenobarbital Elixir and Elixir Controls.**—Rats were dosed with phenobarbital elixir U. S. P. XIV and appropriate elixir controls as shown in Table II. The elixir produced a very rapid nonphenobarbital-like induction time with death of respiratory arrest following within two hours after administration. Animals receiving only ethanol slept for an average of almost ten hours. Rats receiving the amaranth-glycerol-sucrose blank displayed a true loss of righting reflex, followed

TABLE I.—LOG DOSE SCREEN OF PHENOBARBITAL SODIUM ORALLY TO NEBRASKA STOCK RATS

Dosage, mg /Kg.	Sex	Rats Per Group	Mean Duration Time			
			Inj -LRR, min	LRR-N, hr.	Inj -N, hr	Inj -Death hr <sup>a</sup>
84.09	M	5	..	...	.	...
	F	5	..	.	..	...
100.00	M	5	....	....	.	..
	F	5	164 (2) <sup>b</sup>	9 8 (2)	12.6 (2)	..
118 92	M	5	113 (3)	11.4 (3)	13.3 (3)	.
	F	5	111 (5)	11 3 (5)	13.2 (5)	.
141.42	M	5	69 (2)	14.0 (2)	15 1 (2)	..
	F	5	104 (3)	14.4 (3)	16 1 (3)	..
168.18	M	5	63 (3)	21 8 (3)	22.9 (3)	.
	F	5	87 (5)	21 2 (5)	22.6 (5)	..
200 00	M	5	76 (5)	24 1 (5)	25.3 (5)	.
	F	5	137 (5)	25 5 (5)	27.7 (5)	.
237.84	M	5	49 (5)	≤ 40.7 (5) <sup>c</sup>	≤ 41.5 (5)	..
	F	5	54 (5)	≤ 35 6 (5)	≤ 36 5 (5)	...
282.84	M	5	64 (5)	≤ 40.9 (4)	≤ 42.0 (4)	2.2 (1)
	F	5	44 (5)	≤ 44.2 (4)	≤ 45 0 (4)	3 3 (1)

<sup>a</sup> Death by respiratory arrest<sup>b</sup> Figures in parentheses represent the number of rats upon which the means based.<sup>c</sup> ≤ Designates equal to or less thanTABLE II.—PHENOBARBITAL ELIXIR AND VARIOUS ELIXIR CONTROLS ORALLY TO MALE NEBRASKA STOCK RATS<sup>a</sup>

Test Preparation	Rats Per Group	Mean Duration Time				A F <sup>c</sup>	N F <sup>d</sup>
		Inj -LRR, min	LRR N, hr	LRR-Death, hr <sup>b</sup>	Inj -Death, hr <sup>b</sup>		
Phenobarbital elixir	10	13 (10) <sup>c</sup>		1 5 (10)	1 7 (10)	10	9
15% Ethanol in water	7	7 (7)	9 8 (7)				
Sweet orange peel tincture in water	7						
Amaranth-glycerol-sucrose in water	7	53 (7)		0 2 (7)	1 1 (7)	7	2
Amaranth-sucrose in water	7						
45% Glycerol in water	7	55 (7)		≥ 0 04 (7) <sup>e</sup>	1.0 (7)	5	2
90% Glycerol in water	7	183 (7)	.	≥ 0 07 (7)	3 1 (7)	6	1

<sup>a</sup> Phenobarbital dosage as the elixir was 182.7 mg /Kg Dosage volume for the first six test preparations was 45.7 cc /Kg<sup>b</sup> Death by respiratory arrest Death for all animals receiving glycerol was preceded by clonic convulsions<sup>c</sup> Number of rats with anal flow of test solution noted prior to death<sup>d</sup> Number of rats with nasal flow of test solution after death<sup>e</sup> Figures in parentheses represent the number of rats upon which the mean is based.<sup>f</sup> Dosage volume was 22.8 cc /Kg<sup>g</sup> ≥ designates equal to or greater than

shortly by clonic convulsions and death of respiratory arrest. This activity appeared to be due solely to the presence of glycerol. Decreasing the dosage volume, yet administering an equivalent amount of glycerol produced essentially the same response although induction time was significantly more prolonged. Most animals receiving glycerol displayed a fluid diarrhea soon after dosage. In certain cases this anal discharge was bloody. Concurrent with muscular relaxation after death, fluid was observed flowing from the nostrils of some of these rats. Upon autopsy the lungs were normal in size and color, although distension of the stomach and intestines was noted.

**Phenobarbital and Phenobarbital Sodium in Aqueous Vehicles.**—Male and female rats were orally dosed with either phenobarbital sodium in an aqueous solution, phenobarbital sodium in 0.25% aqueous agar, or phenobarbital finely suspended in 0.25% aqueous agar as shown in Table III, part 1.

Attempts to apply statistics to the raw data revealed that the variances of the groups receiving phenobarbital sodium were not comparable to the variance of the group receiving phenobarbital

Conversion of the raw data into natural log transformation data revealed upon statistical treatment with the *F* test that variances between the groups were then comparable and that the *t* test could be validly applied to this data. This finding further indicated that group mean and standard error of the mean determinations should be calculated using log transformation data rather than raw data in order to depict most accurately the research results of this experiment. A group mean calculated from log transformation data is termed a geometric mean. The 95% confidence limits for the geometric mean were calculated from the log transformation data and are two times the standard error of the mean. Statistical calculations followed the format outlined by Bliss and Calhoun (6). Results are summarized in Table III, part 2.

The induction time of rats dosed with phenobarbital was approximately three and one-half times longer than that recorded for the other two groups receiving the salt. Differences in vehicles did not grossly affect induction time for phenobarbital sodium. The pH of the 4% aqueous solution of phenobarbital sodium was 9.1, while that of the 0.25%



TABLE III, PART 1.—PHENOBARBITAL AND PHENOBARBITAL SODIUM ORALLY IN AQUEOUS VEHICLES TO MANOR FARM RATS<sup>a</sup>

Test Preparation	Test Group Code	Sex	Geometric Mean Duration Time		
			Inj.-LRR, min.	LRR-N, hr.	Inj.-Death, hr. <sup>b</sup>
Phenobarbital sodium in distilled water	PNa.W.M	M	27 (10) <sup>c</sup>	28.9 (9)	4.5 (1)
			23-32 <sup>d</sup>	25.7-32.6	
	PNa.W.F	F	31 (10)	34.3 (8)	4.5 (2)
			25-39	32.6-36.2	
Phenobarbital sodium in 0.25% agar	PNa.A.M	M	30 (10)	28.5 (10)	...
			24-36	26.5-30.6	
	PNa.A.F	F	39 (10)	34.0 (8)	5.0 (2)
			34-46	31.5-36.6	
Phenobarbital in 0.25% agar	P.A.M	M	116 (10)	27.5 (10)	...
			92-145	25.0-30.2	
	P.A.F	F	126 (10)	33.4 (10)	...
			94-170	31.8-35.0	

<sup>a</sup> Dosage used was 200.0 mg./Kg. for phenobarbital sodium and 182.7 mg./Kg. for phenobarbital. Ten rats were dosed per sex per group.  
<sup>b</sup> Death by respiratory arrest.  
<sup>c</sup> Figures in parentheses represent the number of rats upon which the mean is based.  
<sup>d</sup> Range figures indicate the 95% confidence limits of the geometric mean. These figures are calculated using log transformation data.

TABLE III, PART 2.—TESTS OF SIGNIFICANCE

Comparison <sup>a</sup>	Test, Observed P	
	Inj.-LRR	LRR-N
PNa.W.M vs. PNa.A.M	>0.50	>0.50
PNa.W.M vs. PNa.A.M	<0.001	>0.50
PNa.W.F vs. PNa.A.F	0.10-0.05	>0.50
PNa.W.F vs. P.A.F	<0.001	0.50-0.25
PNa.W.M vs. PNa.W.F	0.50-0.25	0.025-0.01
PNa.A.M vs. PNa.A.F	0.05-0.025	0.005-0.001
P.A.M vs. P.A.F	>0.50	0.005-0.001
PNa.W.M and PNa.A.M vs. P.A.M	<0.001	0.50-0.25
PNa.W.F and PNa.A.F vs. P.A.F	<0.001	>0.50
PNa.W.M and PNa.A.M vs. PNa.W.F and PNa.A.F	0.05-0.025	<0.001

<sup>a</sup> See Table III, part 1 for test group code names.

agar solution was 8.8. The 3.65% solution-suspension of phenobarbital in 0.25% agar had a pH of 7.2. The slower onset noted with phenobarbital may be due to a difference in pH. Brodie and co-workers (7) postulated that acidic drugs are rapidly absorbed in their lipid soluble nonionized state from the highly acid stomach and are not well absorbed in their lipid insoluble ionized state from the alkaline intestine. Discrepancies in this concept were explained by invoking the Meyer-Overton partition coefficient (8, 9). The gastrointestinal and blood-brain barriers are considered to be primarily lipid in nature. According to the Brodie concept the alkaline preparations of phenobarbital sodium should be poorly absorbed from the stomach, while the neutral solution-suspension of phenobarbital should be well absorbed. Passage of all three preparations into the alkaline intestine should hinder absorption. As

a result of this, the animals dosed with the neutral solution-suspension should have a much shorter onset of narcosis. Using intact laboratory animals in a standardized clinical test situation, this study clearly indicated the opposite situation. Rate of solution for phenobarbital in the gastrointestinal contents may explain the results observed here. Table III, part 2, shows that there is a highly significant sex difference in regard to duration of narcosis, the females sleeping about five hours longer. The pooled data of the two groups receiving phenobarbital sodium indicated that there was also a significant sex difference as to onset of LRR, with the males showing a shorter induction time.

Phenobarbital Sodium to Mice.—In order to determine whether the sex difference previously unreported for long acting phenobarbital would be observed in another species, mice were dosed orally with 200 mg./Kg. of phenobarbital sodium using a 1% aqueous solution. Results are summarized in Table IV. Females had a significantly faster induction time (P 0.05-0.025) and a greater mortality from respiratory arrest as compared with the males. There was no significance (P >0.50) between the sexes as to duration of lost reflex observed with survivors. This indicated a species variation in response.

TABLE IV.—200 MG./KG. PHENOBARBITAL SODIUM ORALLY TO MICE

Sex	Mice Per Group	Geometric Mean Duration Time		
		Inj.-LRR, min.	LRR-RRR, hr.	Inj.-Death, hr.
M	21	25 (21) <sup>a</sup>	18.3 (19)	31.2 (2)
		19-34	13.7-24.5	
F	21	18 (21)	17.8 (15)	35.1 (6)
		16-20	14.0-22.5	

<sup>a</sup> Figures in parentheses represent the number of mice upon which the mean is based, while the range figures indicate the 95% confidence limits for the geometric mean.

Phenobarbital Sodium in Various Sucrose Syrups.—A 10% sucrose solution is considered to be isotonic. To test the effect of hypotonic, isotonic, and hypertonic sucrose solutions upon the response

to phenobarbital sodium, 10 animals per sex, per test treatment were dosed orally as shown in Table V, part 1. Statistical evaluation is summarized in Table V, part 2.

With increasing concentration of sucrose in the vehicle there was a progressive lengthening of induction time. Also, there was a highly significant sex variation in regard to duration of narcosis, with the females sleeping up to eight hours longer than the males. As viscosity and surface tension may be the factors modifying the response in this experiment, these physical properties were measured using the Du Nouy tensiometer and the Ostwald pipet, and are correlated with induction time in Fig 1. Each point in Fig 1 represents the mean of five determinations made at 37°. Qualitatively the viscosity and induction time curves were similar, while the surface tension curve was qualitatively different. This indicated that viscosity was the major contributing factor for the observed lengthening of induction

TABLE V, PART 1—200 MG./KG PHENOBARBITAL SODIUM ORALLY IN VARIOUS SUCROSE SYRUPS TO NEBRASKA STOCK RATS<sup>a</sup>

Test Vehicle	Sex	Geometric Mean Duration	
		Inj -LRR, min	LRR-N, hr
Water	M	42 (32-54) <sup>b</sup>	19.9 (15.9-24.9)
	F	37 (32-44)	26.3 (24.5-28.3)
5% Sucrose	M	43 (37-51)	22.0 (18.8-25.8)
	F	48 (39-60)	22.6 (19.8-25.8)
10% Sucrose	M	45 (40-51)	20.0 (17.8-22.3)
	F	54 (46-63)	24.9 (21.8-28.4)
20% Sucrose	M	48 (41-55)	21.3 (18.1-24.9)
	F	57 (43-75)	24.5 (22.2-27.0)
40% Sucrose	M	57 (39-82)	16.5 (10.3-26.3)
	F	77 (51-111)	24.5 (19.4-31.0)
80% Sucrose	M	94 (67-133)	18.9 (14.9-23.9)
	F	86 (63-118)	24.2 (20.6-28.4)

<sup>a</sup> Ten rats were dosed per sex per test treatment

<sup>b</sup> Figures in parentheses indicate the 95% confidence limits of the geometric mean

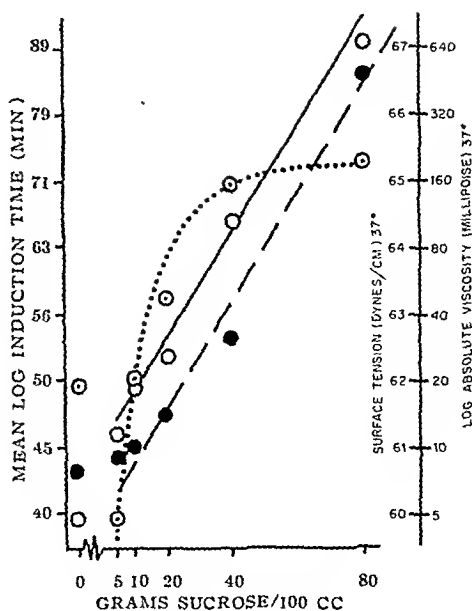


Fig. 1—Relationship of induction time after 200 mg./Kg phenobarbital sodium to the viscosity and surface tension of various sucrose syrup vehicles: O, solid line, induction time; ●, dash line, viscosity; ○, dotted line, surface tension

time. In this instance viscosity parallels osmotic pressure.

**Phenobarbital Sodium at Two Dosage Volumes in Water and 80% Sucrose Syrup.**—Female rats were dosed as shown in Table VI, part 1. Statistical evaluation of the data is summarized in Table VI, part 2. When 200 mg./Kg phenobarbital sodium was administered in aqueous solution it was more rapidly effective from a dilute (2%) rather than from

TABLE V, PART 2—TESTS OF SIGNIFICANCE

Comparison	Inj LRR		LRR-N	
	Between Vehicles	Between Sexes	Between Vehicles	Between Sexes
Water vs				
5% Sucrose	0.10-0.05	>0.50	>0.50	0.05-0.025
10% Sucrose	0.025-0.01	>0.50	>0.50	<0.001
20% Sucrose	0.025-0.01	>0.50	>0.50	<0.001
40% Sucrose	0.005-0.001	>0.50	0.50-0.25	0.025-0.01
80% Sucrose	<0.001	0.50-0.25	0.50-0.25	<0.001

TABLE VI, PART 1—EFFECTS OF DIFFERENT DOSAGE VOLUMES ON RESPONSE OF 200 MG./KG PHENOBARBITAL SODIUM ORALLY TO FEMALE NEBRASKA STOCK RATS<sup>a</sup>

Dosage Volume cc/Kg	Vehicle	Test Group Code	Geometric Mean Duration Time	
			Inj -LRR, min	LRR-N, hr
5.0	Water	PNa W 5	49 (10) <sup>c</sup>	27.3 (9)
			32-74 <sup>d</sup>	26.2-28.5
10.0	Water	PNa W 10	29 (10)	28.5 (7)
			24-35	27.8-29.3
5.0	80% Sucrose	PNa S 5	80 (10)	23.2 (10)
			60-106	17.8-30.3
10.0	80% Sucrose	PNa S 10	129 (10)	23.1 (9)
			106-158	20.7-25.7

<sup>a</sup> Ten rats were dosed per test treatment

<sup>b</sup> Death by respiratory arrest

<sup>c</sup> Figures in parentheses represent the number of rats upon which the mean is based.

<sup>d</sup> Range figures indicate the 95% confidence limits of the geometric mean

TABLE VI, PART 2—TESTS OF SIGNIFICANCE

Comparison	Test, Observed P	
	Inj - LRR	LRR-N
PNa W 5 vs PNa W 10	0 05-0 025	0 50-0 25
PNa S 5 vs. PNa S 10	0 025-0 01	>0 50
PNa W 5 vs PNa S 5	0 10-0 05	0 50-0 25
PNa W 10 vs PNa S 10	<0 001	0 01-0 005

a concentrated (4%) solution, as shown by a significantly shorter induction time and a 30% incidence of fatalities. However, when large concentrations of sucrose in the vehicle rendered phenobarbital's contribution to osmotic pressure insignificant, the effects of administering a more dilute solution of phenobarbital sodium was actually reversed, as shown by a prolonged induction time and a significantly reduced duration of narcosis. This indicated that while the osmotic pressure of the vehicle can modify response, the dosage volume is also an important factor.

**Phenobarbital in Ethanol-Glycerol-Water Mixtures.**—From the data of Krause and Cross (10) various combinations of ethanol and glycerol were selected in which 182.7 mg./Kg. of phenobarbital could be dosed at 5 cc./Kg. With only four rats dosed per test treatment, only qualitative conclusions could be drawn which corroborate the findings of Hazleton and Hellerman (2).

## DISCUSSION

Phenobarbital elixir U. S. P. XIV contains three ingredients with pharmacologic activity: phenobarbital, ethanol, and glycerol. It was impossible to determine the relative merit of the intact elixir because the narcotic effect of phenobarbital was both qualitatively and quantitatively changed by the various actions of ethanol and glycerol. Pharmacologically inactive sucrose was shown to quantitatively alter the phenobarbital response. A proposed new elixir can be formulated on a pharmacologic basis. It should be a dilute hydroalcoholic solution of phenobarbital sodium. The presence of ethanol would lend a certain amount of pharmaceutical stability as well as supplementing and hastening the action of phenobarbital sodium. A synthetic sweetener, such as cyclamate sodium N. N. D. which does not appreciably change viscosity, should be substituted for the sucrose and glycerol. Further flavoring is indicated. This postulated elixir fulfills the classic definition, but would avoid the undesirable vehicle pharmacodynamics found in phenobarbital elixir U. S. P. XIV by this study and others.

## SUMMARY

- Using a constant dosage volume of 5 cc./Kg. the oral narcotic ED<sub>50</sub> for phenobarbital sodium administered to rats was found to be 126 mg./Kg. ( $\pm 11.5$ ). The dosage level adopted for this

study was 200 mg./Kg. or its equivalent, since the ED<sub>95</sub> was calculated to be 190 mg./Kg. The safety index (LD<sub>50</sub>/ED<sub>95</sub>) was estimated to be equal to 1.26.

- The pharmacologic end point of righting reflex regained (RRR) cannot be used to describe accurately the action of long acting barbiturates. A new end point, normalcy, was defined.

- Ethanol and glycerol were shown to modify qualitatively and quantitatively the effect of the phenobarbital in phenobarbital elixir U. S. P. XIV.

- Group mean and standard error of the mean calculations for induction time and duration of barbiturate narcosis must be calculated from common log transformation data to describe experimental results most accurately.

- Phenobarbital sodium was more efficient in inducing narcosis than phenobarbital, when these compounds were administered in aqueous solution-suspensions of low viscosity.

- Increasing concentrations of sucrose in aqueous test solutions of phenobarbital sodium progressively lengthened the induction time for narcosis. This appeared to be a function of viscosity rather than surface tension.

- Phenobarbital sodium was more rapidly effective in dilute aqueous solutions than in concentrated aqueous solutions.

- An equal dosage of phenobarbital sodium was less rapidly effective when administered 10 cc./Kg. than 5 cc./Kg. in an 80% aqueous solution of sucrose.

- There was found a definite sex variation in response to phenobarbital and phenobarbital sodium administered orally to rats of the Wistar strain. Males had a shorter duration of narcosis as well as a somewhat shorter induction time as compared to the females. No colony variation was seen. A qualitative species variation was observed between rats and mice.

## REFERENCES

- (1) Seeberg, V. P., and Dille, J. M., *THIS JOURNAL*, 32, 133 (1943).
- (2) Hazleton, L. W., and Hellerman, R. C., *ibid.*, 35, 161 (1946).
- (3) "U. S. Pharmacopeia," 14th rev., Mack Publishing Co., Easton, Pa., 1950, p. 453.
- (4) Holck, H. C. O., Miya, T. S., Dunham, N. W., and Yim, G. K., "Laboratory Guide in Pharmacology," Burgess Publishing Co., Minneapolis, Minn., 1959, p. 1.
- (5) Miller, L. C., and Tainter, M. L., *Proc. Soc. Exptl. Biol. Med.*, 57, 261 (1944).
- (6) Bliss, C. I., and Calhoun, D. W., "Outline of Biometrics," Yale Co. op. Corp., New Haven, Conn., 1954, p. 87.
- (7) Brodie, B. B., and Hogben, C. A. M., *J. Pharm. and Pharmacol.*, 9, 345 (1957).
- (8) Meyer, H., *Arch. exptl. Pathol. Pharmacol. Naunyn-Schmiedeberg's*, 46, 338 (1901).
- (9) Overton, E., "Studien über die Narkose," Fischer, Jena, 1901.
- (10) Krause, G. M., and Cross, J. M., *THIS JOURNAL*, 40, 137 (1951).

# Substituted Xanthines II\*

## Preparation and Properties of Some (2-Hydroxyalkyl)trimethylammonium Theophyllinates

By FREDERICK VIDAL† and THEODORE I. FAND‡

A new and convenient method has been developed for the large scale preparation of (2-hydroxyethyl)trimethylammonium theophyllinate.<sup>1</sup> The synthesis of several (2-hydroxyalkyl)trimethylammonium theophyllinates was accomplished by the addition of trimethylamine to various epoxyalkanes and then reacting the resulting intermediate with theophylline or 8-substituted theophylline derivatives. Various properties of these compounds are described.

THE METHYL derivatives of xanthine, particularly theophylline, have been used for many years as therapeutic agents in bronchial asthma, angina pectoris, and as mild diuretics. Theophylline has also found clinical application for myocardial stimulation and has been employed occasionally for the treatment of hypertension.

Although the therapeutic value of this drug is well founded, it is not without undesirable side effects. Of these, the most important is that it causes gastric irritation with concomitant nausea and vomiting. Another disadvantage of theophylline is its very limited solubility in water. In order to increase its therapeutic effectiveness, particularly in oral administration, preparations with enhanced water solubility were investigated. Certain double compounds of theophylline with salts and aliphatic amines showed considerably higher solubility. Several such addition products or mixtures have been reported, including aminophylline, theophylline-methylglucamine, and theophylline sodium glycinate (1, 2).

In contrast to the double compounds mentioned above, it was shown that the condensation of choline with theophylline afforded an ionic compound which possessed properties of a true salt (3).

(2-Hydroxyethyl)trimethylammonium theophyllinate was previously prepared by two methods. The first (4) consisted of reacting choline base with an alcoholic suspension of theophylline. The second method (3) involved heat-

ing theophylline with an excess of choline bicarbonate in an aqueous medium until the evolution of carbon dioxide ceased. The reaction product was then obtained upon concentrating the solution under reduced pressure. While these procedures were suitable for laboratory work, they were not satisfactory for large scale operation.

The method of choice (5) for preparing this quaternary ammonium salt was patterned after Wurtz's well-known synthesis (6) of choline with several pertinent modifications. Good yields of several (2-hydroxyalkyl)trimethylammonium theophyllinates, free of inorganic salts, resulted from the reaction between trimethylamine, various epoxyalkanes, and theophylline or its 8-substituted derivatives.

(2-Hydroxyethyl)trimethylammonium theophyllinate was conveniently formed in high yields when ethylene oxide was used in this reaction. In order to determine the optimal reaction conditions, the effects of the following variables were studied: (a) pressure, (b) solvents, (c) temperature, and (d) presence of water. It was found that the yields for (2-hydroxyethyl)trimethylammonium theophyllinate ranged between 83 and 97% of the theoretical under the different conditions employed. The best yield could be obtained by the use of anhydrous isopropanol as solvent and by cooling the starting materials at the beginning of the reaction to  $-10^{\circ}$ . Furthermore, it was possible to carry out the reaction at atmospheric pressure if these conditions were maintained. The reaction was found to be vigorous and exothermic both under completely anhydrous conditions or in the presence of catalytic amounts of water. (2-Hydroxyethyl)trimethylammonium theophyllinate was the sole reaction product in all but one of these experiments. In the latter case, 7-(2-hydroxyethyl)-theophylline was isolated as a minor component in 5% yield, while the major product was the quaternary ammonium salt, obtained in 89% yield. Table I summarizes most of the results obtained in the investigation of the optimal reaction conditions for the preparation of (2-hydroxyethyl)trimethylammonium theophyllinate or its 8-substituted derivatives.

\* Received August 31, 1959, from the Research Laboratories, Nepera Chemical Co., Inc., Yonkers 2, N. Y.

For the preceding paper in this series see Reference 3.

† Present address: Wallace and Tiernan, Inc., Belleville, N. J.

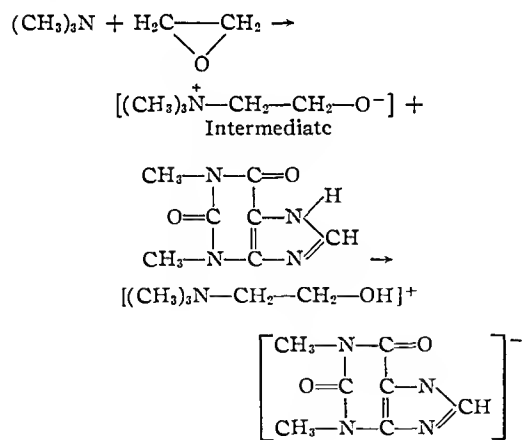
‡ Present address: Warner-Lambert Research Institute, Morris Plains, N. J.

<sup>1</sup> Cholelodyl is the registered trade mark of Warner Lambert Pharmaceutical Co., for its brand of choline theophyllinate

If the 8-bromo-, chloro-, or nitro-theophylline derivatives were employed, the sole reaction product isolated in each instance, was (2-hydroxyethyl)trimethylammonium 8-bromotheophyllinate, (2-hydroxyethyl)trimethylammonium 8-chlorotheophyllinate, and (2-hydroxyethyl)trimethylammonium 8-nitrotheophyllinate, respectively.

The reaction of theophylline with propylene oxide and trimethylamine led to the formation of only one product, namely, (2-hydroxypropyl)trimethylammonium theophyllinate, obtained in a yield of 67%. However, when 1,2-epoxybutane was used instead of 1,2-epoxypropane, two products were isolated; namely, (2-hydroxybutyl)trimethylammonium theophyllinate (in 48% yield) and 7-(2-hydroxybutyl)-theophylline (in 26% yield).

On the basis of our findings, the formation of (2-hydroxyethyl)trimethylammonium theophyllinate is postulated to proceed as follows: in the initial step trimethylamine reacts with the reactive ethylene oxide by opening the oxirane ring. The resultant intermediate then reacts with theophylline to give the final product. A possible mechanism for the reaction, under anhydrous conditions, may be represented schematically as follows:



In the presence of water, however, it is assumed that the intermediate first formed is choline, which then condenses with theophylline, yielding the desired quaternary ammonium salt.

Several attempts were made to prepare (1-methyl-2-hydroxypropyl)trimethylammonium theophyllinate and (2-hydroxyphenethyl)trimethylammonium theophyllinate by heating theophylline and trimethylamine with 2,3-epoxybutane and with styrene oxide, respectively. We were unable, however, to obtain the desired quaternary ammonium salts in crystalline form

by the described method. In these instances, the only reaction products isolated and identified were the 7-(1-methyl-2-hydroxypropyl)- and 7-(2-hydroxyphenethyl)- derivatives of theophylline, respectively.

According to our results the yield of the corresponding quaternary ammonium salt decreased as the chain-length of the alkyl group of the epoxide increased, or if an aryl substituted epoxide was used. Simultaneously with the decrease in yields of the quaternary ammonium compounds, there was an increase in the amounts of the 7-(2-hydroxyalkyl)-theophyllines. Obviously, the formation of the latter theophylline derivatives competes with the main reaction and the proportion of these two different types of compounds obtained, depends on the alkyl chain length of the epoxide used.

A future publication will deal with the properties and a convenient procedure for the preparation of some 7-(2-hydroxyalkyl)-theophyllines.

## EXPERIMENTAL<sup>2</sup>

**Materials.**—8-Bromotheophylline was prepared by the method of Biltz and Strufe (7) and 8-chlorotheophylline was prepared according to Fischer and Ach (8). 8-Nitrotheophylline was obtained according to a previously described method (3).

Trimethylamine and the different epoxides were obtained from commercial sources.

**Procedure.**—The general reaction procedure for the preparation of the compounds listed in Table I is illustrated by the following examples. In the experiments in which water was used as the solvent, it was necessary to evaporate the solution *in vacuo* to about one-third of its original volume in order to isolate the reaction products. Isopropanol was then added until a precipitate formed upon cooling.

**(2-Hydroxyethyl)trimethylammonium Theophyllinate.**—A solution of 28 Gm. (0.47 mole) of dry trimethylamine in 100 ml. of anhydrous isopropanol was placed in the reaction flask and cooled to  $-10^\circ$ . Twenty-one grams (0.48 mole) of ethylene oxide was added while stirring, followed by 65 Gm. (0.36 mole) of anhydrous theophylline. During this period the temperature was maintained around  $-10^\circ$ . The slurry formed was stirred vigorously at  $-10^\circ$  for one hour and at room temperature for another hour. During this latter time, an exothermic reaction occurred and the temperature of the mixture rose to  $65^\circ$ . When the reaction subsided, the slurry was stirred at about  $60^\circ$  for an additional one and one-half hours. Upon cooling in ice, the product was filtered on a Büchner funnel and washed thoroughly with cold isopropanol. The yield of (2-hydroxyethyl)trimethylammonium theophyllinate was 99 Gm. (97%). The product melted at  $185\text{--}187^\circ$  and did not depress the melting point of an authentic sample of (2-hydroxyethyl)tri-

<sup>2</sup> All melting points have been corrected and were taken in capillaries.

TABLE I.—REACTIONS OF ETHYLENE OXIDE WITH TRIMETHYLAMINE AND THEOPHYLLINE OR 8-SUBSTITUTED THEOPHYLLINE DERIVATIVES

No.	Trimethyl- amine	Purine Deriva- tive	Solvent, ml.	Pressure	Time, hr.	Reaction Temp. °C.	Products Name	M. P., °C.	Yield, %
1	25% Aqueous solution	Th <sup>a</sup>	100 H <sub>2</sub> O	Sealed tube	48 1	20 100	ChTh <sup>b</sup>	188– 188.5 <sup>c, f</sup>	83.5
2	25% Aqueous solution	Th <sup>a</sup>	100 H <sub>2</sub> O	Atmospheric	48 1	20 100	ChTh <sup>b</sup>	187.5– 188.5 <sup>c, f</sup>	85
3	Anhydrous	Th <sup>d</sup>	220 Isopr. + 20 H <sub>2</sub> O	Atmospheric	2 2	0 60	ChTh <sup>e</sup>	187.5– 188.5 <sup>c, f</sup>	89.5
4	25% Aqueous solution	BrTh <sup>g</sup>	50 H <sub>2</sub> O	Sealed tube	48 1	20 100	ChBrTh	65– 65.5 <sup>f, h</sup>	85.5
5	25% Aqueous solution	ClTh <sup>g</sup>	50 H <sub>2</sub> O	Atmospheric	48 1	20 50	ChClTh	97–99 <sup>f, i</sup>	86
6	Anhydrous	NTh <sup>g</sup>	180 Isopr. + 20 H <sub>2</sub> O	Atmospheric	2 2	0 60	ChNTh	248 <sup>f, j</sup>	87.5

Th = theophylline; BrTh = 8-bromotheophylline; ClTh = 8-chlorotheophylline; NTh = 8-nitrotheophylline; Isopr. = isopropanol; ChTh = (2-hydroxyethyl)trimethylammonium theophyllinate; ChBrTh = (2-hydroxyethyl)trimethylammonium 8-bromotheophyllinate; ChClTh = (2-hydroxyethyl)trimethylammonium 8-chlorotheophyllinate; ChNTh = (2-hydroxyethyl)trimethylammonium 8-nitrotheophyllinate

<sup>a</sup> A 1:1:1 mole ratio of Th, base, and epoxide was used. <sup>b</sup> Unreacted Th, 7%, was also recovered. <sup>c</sup> Reported (3) m. p. 186°. <sup>d</sup> A 0.9:1:1 mole ratio of Th, base, and epoxide was used. <sup>e</sup> In addition to the quaternary ammonium compound, 4.6% of 7-(2-hydroxyethyl)-theophylline, m. p. 162–164° was also obtained in this experiment. <sup>f</sup> Point depression when admixed with an authentic sample of the same product. <sup>g</sup> 0.75:1:1 mole ratio of 8-substituted Th, base, and epoxide was used. <sup>h</sup> Reported (3) m. p. 248°. <sup>i</sup> (3) m. p. 248°.

methyammonium theophyllinate, prepared by another method.

(2-Hydroxypropyl)trimethylammonium Theophyllinate.—In a three-necked flask equipped with a stirrer, gas inlet tube, and an addition funnel was placed 100 ml. of isopropanol. The flask was immersed in an ice bath, 31 Gm. (0.52 mole) of trimethylamine bubbled into the solvent, and 30 Gm. (0.52 mole) of propylene oxide added. To this solution, 72 Gm. (0.4 mole) of anhydrous theophylline was added slowly while stirring over a period of thirty minutes. The slurry which formed was then stirred vigorously at 5° for one hour and at room temperature for one and one-half hours, when the flask was equipped with a condenser in place of the inlet tube. During this latter time, an exothermic reaction took place and the temperature rose to about 60°. Finally, the reaction mixture was stirred at about 60° for an additional one and one-half hours. The slurry was cooled to 0°, the solid was filtered off and washed thoroughly with cold isopropanol. Recrystallization of the white product from isopropanol gave 80.3 Gm. (67.5%) of (2-hydroxypropyl)trimethylammonium theophyllinate, m. p. 186.5–187.5°. A mixed melting point with a sample of (2-hydroxyethyl)trimethylammonium theophyllinate showed a marked depression (m. p. 170–172.5°). (2-Hydroxypropyl)trimethylammonium theophyllinate was found to be very soluble in water (1:1) and a 1% aqueous solution had a pH of about 9.5.

Anal.—Calcd. for C<sub>13</sub>H<sub>21</sub>N<sub>3</sub>O<sub>3</sub>: C, 52.5; H, 7.79; N, 23.56; neut. equiv., 297. Found: C, 52.95; H, 7.6; N, 24.0; neut. equiv., 294.

(2-Hydroxybutyl)trimethylammonium Theophyllinate.—To 50 ml. of isopropanol, cooled to 0°, was added 16 Gm. (0.27 mole) of trimethylamine and 18 Gm. (0.25 mole) of 1,2-butylene oxide while

the temperature was maintained at 0°. This solution was stirred and 36 Gm. (0.2 mole) of anhydrous theophylline was introduced in small portions. After the final addition, the slurry which formed was stirred vigorously at 0° for one hour and at room temperature for an additional two hours. After the first hour, an exothermic reaction ensued and the mixture reached a temperature of 40°. When the reaction had subsided, the mixture was stirred at 60° for another hour. During this time, the suspended material originally present had dissolved. Upon cooling to 5°, the precipitate which formed was collected on a Büchner funnel and washed thoroughly with cold isopropanol, m. p. 150–154°. Two recrystallizations of the crude material from isopropanol gave 30.2 Gm. (48.5%) of (2-hydroxybutyl)-trimethylammonium theophyllinate, m. p. 164–166°. This salt was somewhat hygroscopic, very soluble in water, and a 1% aqueous solution gave an alkaline reaction (pH 9.5).

Anal.—Calcd. for C<sub>14</sub>H<sub>25</sub>N<sub>3</sub>O<sub>3</sub>: N, 22.5. Found: N, 22.9.

After concentrating the combined mother liquors to a small volume and subsequent cooling, 24 Gm. of a white crystalline precipitate, m. p. 132–137°, separated. This fraction was twice recrystallized from isopropanol to yield 13 Gm. (25.8%) of 7-(2-hydroxybutyl)-theophylline, m. p. 141–142.5°.

Anal.—Calcd. for C<sub>11</sub>H<sub>16</sub>N<sub>3</sub>O<sub>3</sub>: N, 22.22. Found: N, 22.6.

## SUMMARY

1. A series of (2-hydroxyalkyl)trimethylammonium theophyllinates has been prepared for evaluation of diuretic and antispasmodic activities.

2. A new and more convenient procedure for the synthesis of these compounds has been described.

\* Microanalyses for C and H were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.; N analyses were carried out by Miss Ruth Becker; the neutral equivalent determination was made by Mr. Milton Geller.

3 The following new compounds have been characterized: (2 - hydroxypropyl)trimethylammonium theophyllinate, (2-hydroxybutyl)-trimethylammonium theophyllinate, and 7-(2-hydroxybutyl)-theophylline

4 The compounds tested showed comparatively low toxicity. Previous research has disclosed effective bronchodilator action of some of these compounds

## REFERENCES

- (1) Martin, E. W., and Cook, E. F., "Remington's Practice of Pharmacy," 11th ed., The Mack Publishing Co. Easton, Pa., 1956, pp. 906-907, 911-912
- (2) Osol, A., and Farrar, C. E., "The Dispensary of the United States of America," 25th ed., J. B. Lippincott Co. Philadelphia, Pa., 1955, pp. 65-68, 1410-1413, 1898
- (3) Duesel, B. F., Berman, H., and Schachter, R. J., *THE JOURNAL*, 43, 619(1954)
- (4) Feinstein, W. H., U. S. pat. 2,667,487, 1953
- (5) Fand, T. I., and Vidal, F., U. S. pat. 2,776,288 1957
- (6) Wurtz, A., *Ann. Suppl.*, 6, 200(1868), Meyer, K. H. and Hopff, H., *Ber.*, 54, 2274(1921)
- (7) Biltz, H., and Strufe, K., *Ann.*, 402, 136(1914)
- (8) Fischer, E., and Ach, F., *Ber.*, 39, 423(1906)

## A Comparison of the Effects of Colchicine and Some Purified Veratrum Alkaloids on Nuclear Division in Roots of *Allium cepa* L.\*

By DOUGLAS L. SMITH† and L. DAVID HINER

Purified alkaloidal mixtures and crystalline veratrum alkaloids were compared with colchicine as to effects on nuclear and cellular division. Chromosomal aberrations often resulted but appeared to be due to a different type of action.

THE REMARKABLE EFFECTIVENESS of colchicine to alter the nuclear mitotic processes in meristematic tissues is well established (1). Living cells respond almost universally to colchicine after a basic pattern which constitutes the colchicine mitosis (c-mitosis), in which selective inhibition of spindle fiber formation interferes with nuclear division at metaphase.

Many chemical agents other than colchicine have been used to produce nuclear and cytoplasmic changes in plants (2-9). In 1944, Witkus and Berger reported on "veratrine, a new polyploidy inducing agent" (10). According to these authors the cytological effects of this agent were similar to those of colchicine with a few

differences in the mechanism of action by which polyploidy was produced. Because of the renewed interest in the active principles of veratrum and related genera (Zygadenus) by the medical profession within recent years, numerous investigators have attempted to isolate new veratrum-like extracts (11-13), to characterize the isolated derivatives (14-16), and to evaluate what, if any pharmacological activity resides in the agents thus obtained (17-19). With this renewed interest in *Veratrum viride* and related derivatives, many purified alkaloids heretofore unobtainable have now become available. Because of the close structural similarity between the alkaloids in veratrine and the alkaloids found in *Veratrum viride* Ait. and *Veratrum album* L., it was thought important to conduct a study to determine (a) whether some purified alkaloidal mixtures (Veriloid<sup>1</sup>) or crystalline alkaloids of veratrum (protoveratrine A<sup>2</sup>) possess the ability to modify the cycle of nuclear and cellular division, and (b) to determine how such activity compares in kind and degree to that already established for colchicine. The effects of colchicine on nuclear

\* Received August 21, 1959, from the University of Utah, College of Pharmacy, Salt Lake City.  
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

This work was submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Pharmacy (Pharmacognosy).

† Currently serving on active duty as instructor, Department of Chemistry, United States Air Force Academy, Colorado.

Appreciation is expressed for help in the laboratory and with the manuscript to the several members of the staffs of the University of Utah College of Pharmacy, the Departments of Botany and Genetics of the University College, and the Department of Bacteriology of the College of Medicine.

<sup>1</sup> Veriloid, a mixture of active principles from *Veratrum viride* Ait. standardized for hypotensive activity to reference standard alkaverin, supplied by Dr. J. E. Campion, Riker Laboratories Inc., Los Angeles, Calif.

<sup>2</sup> Protoveratrine A, a purified alkaloidal fraction obtained from *Veratrum album* L., supplied by Dr. J. E. Campion, Riker Laboratories Inc., Los Angeles, Calif., and Dr. L. C. Weaver, Pitman Moore Company, Indianapolis, Ind.

mitotic activity were investigated because this agent has become the agent of choice for experimental alteration of cell division processes and because it was desired to establish a comparison standard for subsequent studies on Veriloid and protoveratrine A. The results of this investigation provide the basis for this report.

## METHODS

Onion bulbs, *Allium cepa* L., var. White Portugal, were used as the experimental plant. A solution of 2% 2-chloroethanol<sup>1</sup> in tap water was used to induce sprouting of new roots on the bulbs. The purpose of soaking the bulbs in the 2-chloroethanol solution was to induce the sprouting of more roots than would result from the use of tap water alone (20). After the roots had sprouted, constant solution temperatures at  $30 \pm 0.5^\circ$  were maintained at all times. Root tips were prepared for microscopic examination by standard fixing, staining, and mounting procedures (21-24).

All intact cells observed in three scannings through the upper, middle, and lower third of a slide were recorded and the per cent of cells in any stage of nuclear mitotic division was calculated. Statistical analyses of the experimental data were performed by utilization of the analysis of variance technique after minor transformation of the original data to angles (25, 26).

For both colchicine and Veriloid, a population sample of 100 allium bulbs was randomly divided into 5 main groups of 20 bulbs each. Each of the main groups was further randomized and subdivided into 4 subgroups of 5 bulbs each. The 5 main groups consisted of 4 concentrations of the drug from 0.1 to 0.8% (concentrations represented colchicine U. S. P. or Veriloid equivalent to reference standard alkavervir). The remaining main group served as the control. The subgroups represented four different times of exposure to the requisite test solution, i.e., three, six, nine, or twelve hours.

In order to dissolve the Veriloid, a minimum amount of diluted acetic acid N. F. (1.4 ml./100 ml. of solution) was employed. This amount of acid (equivalent to 0.084 Gm. of  $C_2H_4O_2$ ) was found by examination of the cells to be noninjurious to the root tissues. Special glass holders for the bulbs and alkaloidal solutions were constructed from Pyrex glassware (Fig. 1) which would hold approximately

2 to 4 ml. of solution. Each glass holder consisted of a watch glass to which a piece of glass tubing, sealed at one end, was heat-fused with simultaneous vacuum suction. The holders were suspended on a wire rack in the constant temperature bath.

In investigating the effects of protoveratrine A, a population sample of 25 allium bulbs was used and randomized into 5 groups of 5 bulbs each. For this agent, the groups represented 4 different times of exposure to an acidified 0.025% solution and a control group containing no protoveratrine.

## RESULTS

The effects of different concentrations of colchicine and different times of exposure on nuclear mitotic division are listed in Table I and the statistical analysis of these data is shown in Table II. The analysis indicated that colchicine treatment significantly decreased the number of mitotically dividing nuclei ( $F=5.18$ ;  $P<0.01$ ). Further, the individual factors contributing to the total treatment effects (time, concentration, and interaction) all showed significant changes (ratios for the concentration and interaction factors were highly significant).

In Table I, examination of individual means (MNDN) and group means (GM) indicates that for the concentration factor alone (upper portion), all colchicine concentrations employed in this study decreased the total number of dividing nuclei as represented by the GM values when compared with the GM obtained for the control. Except for the 0.2% concentration, the GM values were significantly different than the control value. Except for the twelve-hour exposure to the 0.2% colchicine concentration, the MNDN values for exposure times of nine and twelve hours decreased as the concentration of colchicine increased ( $P<0.05$  for the nine- and twelve-hour exposures to 0.4% and 0.8% colchicine). Also, for the colchicine concentrations of 0.4% and 0.8%, MNDN values decreased as the time of exposure was increased from three to twelve hours. There was little effect produced on MNDN values either by increasing the colchicine concentration for the three- and six-hour exposure times or by increasing the time of exposure at colchicine concentrations of 0.1% and 0.2%.

With time as the major variable (Table I, lower portion), MNDN values indicate more clearly that for exposure times of nine and twelve hours, a decrease in the number of dividing nuclei resulted as the concentration of colchicine was increased. A comparison of the differences between group means for the six-hour time exposure and any of the other times by the "Student"  $t$  test indicated that the differences were significant. The high GM value for the six-hour exposure time (3.55%) was primarily due to the high individual MNDN value of 5.3% obtained for the 0.8% colchicine concentration (also see Fig. 2, six-hour exposure time). Since this value failed to follow the general trend established for the other concentrations and exposure times, an additional experiment on roots exposed to 0.8% colchicine for a six-hour time period was indicated. Results obtained from an additional 20 allium bulbs showed the MNDN to be 3.2%; therefore, the 5.3% value obtained in the original experiment was assumed to be aberrant and for further graphical purposes (Fig. 2) the 3.2% value was used.

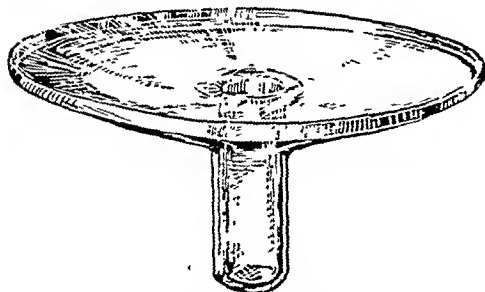


Fig. 1.—Glass solution holder.

<sup>1</sup> Supplied by Distillation Products Industries, Rochester 3, N. Y.



TABLE I.—EFFECTS OF COLCHICINE ON NUCLEAR DIVISION IN ALLIUM ROOTS

Time, hr.→	Main Groups, Concentration																							
	Control				0.1%				0.2%				0.4%				0.8%							
MNDN, <sup>a</sup>	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12				
%	3.9	3.6	4.5	4.0	2.5	2.6	2.6	2.6	3	1	3	6	2.4	4.1	1.9	2.7	2.1	0.9	2.4	5.3	1.5	0.9		
GM, <sup>b</sup> %	4.00				2.58				3.30				1.90				2.53							
Colchicine, %→	Main Groups, Time																							
	Control				3 hr				6 hr.				9 hr.				12 hr.							
MNDN, <sup>a</sup>	.	..	..	.	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8				
%	3	9	3.6	4	5	4.0	2	5	3	1	1.9	2.4	2.6	3.6	2.7	5.3	2.6	2.4	2.1	1.5	2.6	4.1	0.9	0.9
GM, <sup>b</sup> %	4 00				2.48				3.55				2.15				2.13							
<sup>a</sup> Mean number of dividing nuclei <sup>b</sup> Group means.																								

TABLE II.—ANALYSIS OF VARIANCE<sup>a</sup> OF COLCHICINE EFFECTS ON NUCLEAR MITOTIC DIVISION IN ALLIUM ROOTS

Adjustment for Mean		9308.00				Experimental F Ratio	
Nature of Variation		Degrees of Freedom	Sum of Squares		Mean Square		5.18 <sup>b</sup>
Between treatments		19	..	508.31	..	26.753	
Different levels of concentration		..	4	..	235.29	..	
Different levels of time		..	3	..	44.80	..	
Interaction		..	12	..	228.22	..	
Error		80	..	413.73	..	5.172	3.68 <sup>b</sup>
Total		99	..	922.04	..	..	..

<sup>a</sup> Transformation of data to angles.    <sup>b</sup>  $P < 0.01$     <sup>c</sup>  $P < 0.05$ .

TABLE III.—EFFECTS OF VERILOID ON NUCLEAR DIVISION IN ALLIUM ROOTS

Time, hr.→	Main Groups, Concentration																			
	Control				0.1%				0.2%				0.4%				0.8%			
MNDN, <sup>a</sup>	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12	3	6	9	12
%	5.1	4.9	5.5	5.9	4.5	3.4	7.6	8.9	5.7	5.5	7.8	7.3	7.1	5.9	7.3	6.9	6.6	5.9	7.6	6.7
GM, <sup>b</sup> %	5.35				6.10				6.58				6.80				6.70			
Veriloid, <sup>c</sup> %→	Main Groups, Time																			
	Control				3 hr.				6 hr.				9 hr.				12 hr.			
MNDN, <sup>a</sup>	..	..	..	..	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8	0.1	0.2	0.4	0.8
%	5.1	4.9	5.5	5.9	4.5	5.7	7.1	6.6	3.4	5.5	5.9	5.5	7.6	7.8	7.3	7.6	8.9	7.3	6.9	6.7
GM, <sup>b</sup> %	5.35				5.98				5.08				7.58				7.45			

<sup>a</sup> Mean number of dividing nuclei.    <sup>b</sup> Group means.    <sup>c</sup> Alkavervir (Veriloid) reference standard.

TABLE IV.—ANALYSIS OF VARIANCE<sup>a</sup> OF VERILOID EFFECTS ON NUCLEAR DIVISION IN ALLIUM ROOTS

Adjustment for Mean		20899.907				Experimental F Ratio	
Nature of Variation		Degrees of Freedom	Sum of Squares		Mean Square		2.46 <sup>b</sup>
Between treatments		19	..	239.309	..	12.595	
Different levels of concentra- tion		..	4	..	42.854	..	
Different levels of time		..	3	..	113.166	..	
Interaction		..	12	..	83.289	..	
Error		80	..	410.198	..	5.128	1.35
Total		99	..	649.507	..	..	..

<sup>a</sup> Transformation of data to angles.    <sup>b</sup>  $P < 0.01$ .    <sup>c</sup>  $P < 0.05$ .

The effects of different concentrations of Veriloid and different times of exposure on nuclear mitotic division are listed in Table III and the statistical analysis of these data is shown in Table IV. The analysis indicated that, in contrast to the colchicine effects, Veriloid treatment significantly increased the number of mitotically dividing nuclei ( $F=2.46$ ;  $P<0.01$ ). Further, of the individual factors contributing to the total treatment effects, only the time factor indicated a significant change ( $F=7.36$ ;  $P<0.01$ ). Therefore, although the effects of both concentration and time were important for colchicine produced changes, the length of time in which roots were exposed to Veriloid appeared to be of

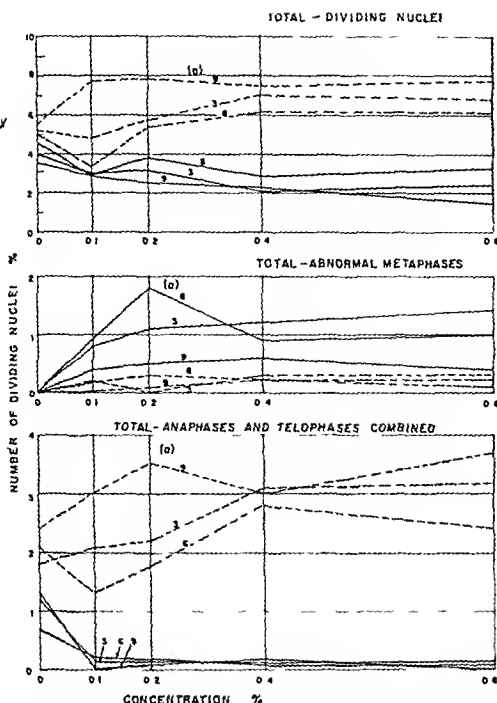


Fig. 2.—Effects of colchicine and Veriloid on nuclear division in *Allium* roots. (a), Hours of exposure; —, colchicine; --- Veriloid.

greater importance to nuclear mitotic division than were the concentrations employed.

Examination of the means in Table III indicates that for the concentration factor alone (upper portion) Veriloid, as employed in this study, had no significant effect on the total number of dividing nuclei when GM values were compared with the value obtained for the control.

With time as the major variable (Table III, lower portion), MNDN values indicate a general increase as the Veriloid concentrations are raised ( $P < 0.01$ ). The difference between GM values for the three-hour and twelve-hour exposure times, 5.98% and 7.45%, respectively, was significant ( $P < 0.05$ ). Further, the most marked increase in the total number of dividing nuclei occurred between the six- and nine-hour exposure times and the difference between these GM's was highly significant ( $P < 0.01$ ).

Figure 2 shows graphically the effects of colchicine and Veriloid on nuclear mitotic division in allium roots for three of the four exposure times studied; three, six, and nine hours. Effects produced by the twelve-hour exposure period were similar to the other exposure times and for purposes of simplification, they are not depicted. In general, the total number of dividing nuclei (upper graph) decreases with increasing concentrations of colchicine whereas for Veriloid, the dividing nuclei remained relatively unaffected (a slight but insignificant increase was the overall effect). The decrease in the total number of dividing nuclei at the concentrations of colchicine studied indicated that the agent was inhibitory at and above concentrations of 0.1%. Typical mitotic effects were produced by the colchicine. At

the three-hour exposure time, the number of abnormal metaphases (middle graph) was observed to increase with each increase in colchicine concentration. Maximum effects were observed to occur at the three-hour exposure to 0.8% colchicine and at the six-hour exposure to 0.2% colchicine. At the three-hour exposure time to 0.8% colchicine, 1.4% of the total 2.5% of dividing nuclei showed abnormal metaphases (56% of the total number of dividing nuclei observed). For Veriloid, the "piling up" effect at metaphase, as noted with colchicine, was not observed.

In the course of investigating the effects of protoveratrine A on nuclear mitotic division, it became apparent that the changes produced were very nearly the same as those observed for Veriloid. For this reason, the investigation of protoveratrine A was not as extensive as it was for colchicine and Veriloid. The effects of different exposure times to 0.025% protoveratrine A on nuclear mitotic division are shown graphically in Fig. 3. The total number of dividing nuclei remained relatively unaltered by different times of exposure to the protoveratrine A. A slight but insignificant ( $P > 0.05$ ) increase over that observed for the control value may be the overall effect produced. The number of abnormal metaphases increased considerably for different times of exposure. At the six-hour exposure time, 1% of the observed nuclei showed abnormal metaphases in the same manner seen in the Veriloid study. Therefore, treatment of the allium roots with protoveratrine A produced mitotic nuclear changes although the number of mitotic dividing nuclei remained relatively unaffected.

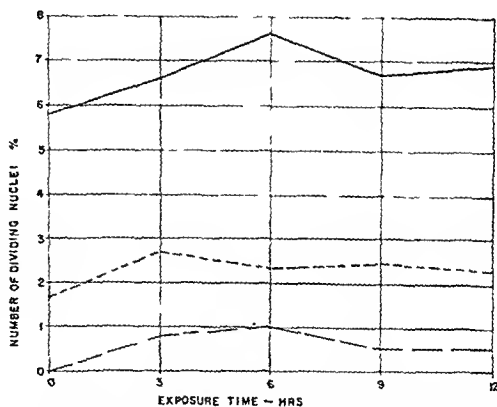


Fig. 3.—Effects of protoveratrine A on nuclear division in *Allium* roots. — Total, dividing nuclei; --- total, anaphases and telophases combined; ..... total, abnormal metaphases.

## DISCUSSION

Colchicine, Veriloid, and protoveratrine A all produced polyploidy in allium roots, although, in general, the effects were much more pronounced after colchicine treatment. A comparison of the effects produced by the veratrum derivatives on nuclear mitotic division with those observed for colchicine revealed many interesting similarities and differences. The equatorial plate was completely absent in the colchicine-treated root tips, whereas

approximately 60% of all metaphases observed in the veratrum-treated root tips showed chromosomes in regular alignment in the equatorial region. It was impossible to differentiate between the two veratrum agents employed solely on the basis of the observable chromosome characteristics, however, the chromosomes of the veratrum-treated cells differed markedly from those treated with colchicine. Veratrum induced "sticky" or angled chromosomes and anaphase bridges were very prominent.

Colchicine treatment decreased significantly ( $P < 0.05$ ) the overall number of dividing nuclei in cells, irrespective of concentration or time of exposure. Because this agent induces metaphasic arrest and yet has no effect on stopping initial mitotic activity, it probably indicates that the concentrations employed were too high to produce an optimum mitotic effect. The most marked differences between colchicine and the veratrum agents were noted with the anaphase and telophase stages (lower graph, Fig. 2). Colchicine treatment caused a great reduction, and sometimes a complete absence, of the number of anaphases and telophases. On the other hand, many anaphases and telophases were to be observed after treatment with the veratrums.

Thus, it appears that alkaloidal agents (mixtures or crystalline) from *Veratrum viride* Ait. and/or *Veratrum album* L. having structural similarities to alkaloids obtained from sabadilla seeds (*Schoenocaulon officinale* A. Gray), may produce polyploidy in roots of *Allium cepa* by an action similar to that of veratrine (10). This action is markedly different and apparently inferior to that seen in colchicine-treated roots and suggests that the derivatives of veratrum do not exert their effects specifically on the mitotic apparatus *per se* as does colchicine, but possibly act by some modification of cytoplasmic function.

### SUMMARY

An attempt has been made to determine whether some purified alkaloidal mixtures or crystalline alkaloids of veratrum possess the ability to modify the cycle of nuclear and cellular division, and to determine how such activity compares in kind and degree to that already established for colchicine. Root growth, preparation, and cell observations were accomplished by standard techniques. The per cent of cells in the process of nuclear division at the time of observation was calculated and results were subjected

to statistical analysis. Drugs were tested in concentrations of 0.025 to 0.8 per cent for predetermined time periods varying from three to twelve hours. Typical colchicine mitotic (c-mitotic) effects were produced with all colchicine concentrations employed. Successful inhibition of the spindle apparatus was achieved and polyploid cells were prominent. Both Veriloid and protoveratrine A produced frequent chromosomal aberrations although their appearance suggested a markedly different mode of action than that observed with colchicine. Polyploid cells were noted and the effects on nuclear mitotic division were similar to those produced by veratrine, an alkaloidal mixture whose constituents are structurally similar to those of the Veriloid and protoveratrine A employed in this study.

### REFERENCES

- (1) Eigsti, O. J., and Dustin, P., Jr., "Colchicine—in Agriculture, Medicine, Biology, and Chemistry," Iowa State College Press, Ames, Iowa, 1955.
- (2) Dreyfus, A., and Zaccaro, O., *Compt. rend. soc. biol.*, 112, 1507(1933).
- (3) Patahzade, F. A., *Compt. rend. acad. sci. U. R. S. S.*, 22, 180(1939).
- (4) Mangenot, G., and Carpentier, S., *Compt. rend. soc. biol.*, 138, 1232(1944).
- (5) Steinegger, E., and Levan, A., *Heredity*, 33, 515(1947).
- (6) Cornman, I., *J. Exptl. Biol.*, 23, 292(1947).
- (7) Novick, A., and Sparrow, A. H., *J. Heredity*, 40, 13(1949).
- (8) Ferguson, J., Hawkins, S. W., and Doxey, D., *Nature*, 165, 1021(1950).
- (9) Wilson, G. B., *J. Heredity*, 41, 226(1950).
- (10) Witkus, E. R., and Berger, C. A., *ibid.*, 35, 131(1944).
- (11) Poetsch, C. E., and Parks, L. M., *This Journal*, 38, 522(1949).
- (12) Hennig, A. J., Higuchi, T., and Parks, L. M., *ibid.*, 40, 168(1951).
- (13) Kupchan, S. M., and Deliwala, C. V., *J. Am. Chem. Soc.*, 74, 3202(1952).
- (14) Fried, J., White, H. L., and Wintersteiner, O., *ibid.*, 71, 3260(1949).
- (15) Klohs, M. W., Keller, F., Koster, S., and Malesh, W., *ibid.*, 74, 1871(1952).
- (16) Nash, H. A., and Brooker, R. M., *ibid.*, 75, 1942(1953).
- (17) Swiss, E. D., *J. Pharmacol. Exptl. Therap.*, 104, 76(1952).
- (18) Mosey, L., and Kaplan, A., *ibid.*, 104, 67(1952).
- (19) Klohs, M. W., Arons, R., Draper, M. D., Keller, F., Koster, S., Malesh, W., and Petracek, F. J., *J. Am. Chem. Soc.*, 74, 5107(1952).
- (20) Smith, D. L., and Hiner, L. D., in preparation.
- (21) Warmke, H. E., *Stain Technol.*, 10, 101(1935).
- (22) Belling, J., *Biol. Bull.*, 50, 160(1926).
- (23) Johansen, D. A., "Plant Microtechnique," McGraw-Hill Book Co., New York, N. Y., 1940.
- (24) McClintock, B., *Stain Technol.*, 4, 53(1929).
- (25) Stearman, R. L., *Bacteriol. Rev.*, 19, 160(1955).
- (26) Snedecor, G. W., "Statistical Methods," 4th ed., Iowa State College Press, Ames, Iowa, 1946.

# The Effect of Anticholinergic Compounds on Several Components of Gastric Secretion in Pylorus-Ligated Rats\*

By MORTON E. GOLDBERG† and G. VICTOR ROSSI

A study was made of the effect of seven anticholinergic compounds: atropine, diphenmethanil (Prantal), oxyphenonium (Antrenyl), propantheline (Pro-Banthine), tridihexethide (Pathilon),  $\alpha$ -(*p*-dimethylaminocyclohexyl)benzhydrol salicylate (SCH 3085), and 1-methyl-4-piperidyl benzilate methobromide (SCH 3444), on various factors which have been cited as bearing a relationship to peptic ulcer (gastric fluid volume, acidity, proteolytic activity, lysozyme activity, and mucin content of gastric secretion, and uropepsin activity). The levels of these several components were contrasted in four-hour ("normal") and fifteen-hour (ulcerated) pylorus-ligated rats. Equipotent antisecretory doses of the anticholinergic drugs significantly reduced the proteolytic activity and free acidity, and increased the lysozyme activity and mucin content per ml. of gastric fluid, but did not alter uropepsin activity in four-hour ligated rats. Comparable, but less consistent results were obtained in ulcerated (fifteen-hour) animals.

NO RECOGNIZED single concept satisfactorily explains the development of peptic ulcer. In addition to nervous and psychic influences, which are believed to play a major role, muscle spasm, ischemia, mechanical irritation, decreased tissue resistance, and abnormal levels of the constituents of gastric secretion have been proposed as factors which contribute to erosion of the mucosa and propagation of the ulcer.

Hydrochloric acid and pepsin have frequently been implicated as agents involved in the formation and maintenance of chronic peptic ulcer. Thus, drugs which possess antisecretory or anti-pepsin effects, or both, are regarded as major adjuncts in the treatment of this condition. In addition, abnormal levels of lysozyme (1, 2) and uropepsin (3, 4) activity have been considered of diagnostic or pathognomonic importance in peptic ulcer.

In this study an attempt was made to correlate certain biochemical components, present in gastric juice or urine, which have been considered as bearing a relationship to the etiology of peptic ulcer. The investigation basically included a determination of the effects of several anticholinergic compounds upon the following factors of the gastric juice of pylorus-ligated rats: volume, acidity, proteolytic activity, lysozyme activity, and mucin content. In addition, the excretory rate of the urinary proteolytic enzyme, commonly referred to as uropepsin, was determined.

Five commercially available anticholinergics: atropine, diphenmethanil, oxyphenonium, propantheline tridihexethide, and two experimental compounds:  $\alpha$ -(*p*-dimethylaminocyclohexyl)benzhydrol salicylate (SCH 3085),<sup>1</sup> and 1-methyl-4-piperidyl benzilate methobromide (SCH 3444),<sup>1</sup> were utilized in this evaluation.

## EXPERIMENTAL

**Animals and Experimental Design.**—Male albino Sherman rats (Scott Farms), maintained on Rockland complete rat pellets and water, *ad libitum*, and housed in temperature and humidity controlled quarters, were used throughout these studies. Prior to ligation of the pylorus, all animals were fasted for forty-eight hours but permitted free access to water. During the fasting and experimental periods, the animals were kept in individual metabolism cages designed to minimize coprophagy and avoid contamination of urine with fecal matter. Only those animals weighing between 185 and 215 Gm. after fasting were utilized.

The technique employed for collection of gastric fluid was essentially that described by Shay (5). The data for "normal" animals presented in this report are based on values obtained with the four-hour pylorus-ligated rat. According to Shay (5), evidence of ulceration was observed only once in a series of 300 four-hour pylorus-ligated rats. In this study of more than 700 similar preparations, gastric ulceration was never observed; therefore, for the purpose of this report, the term "normal" appears justified.

Extension of the ligation period results in ulceration of the stomach and the degree of ulceration is related to the duration of the ligation period. Prolonged ligation ultimately results in perforation of the stomach and death of the animal. The data for "ulcerated" animals given in this report are based on values obtained with the fifteen-hour pylorus-ligated rat. This interval was selected on

\* Received September 29, 1959, from the Department of Pharmacology and Therapeutics, University of California, Los Angeles.

† Morton E. Goldberg, Department of Pharmacology and Therapeutics, University of California, Los Angeles.

A preliminary report was presented to The Scientific Section, A. Ph. A., Los Angeles meeting, April 1958.

† Present address: Mellon Institute, Pittsburgh, Pa.

<sup>1</sup> Generously supplied by Schering Corp., Bloomfield, N. J.

the basis of preliminary experiments which indicated that periods of less than fifteen hours were not uniformly associated with ulcer formation, whereas ligation for longer intervals resulted in excessive incidence of perforated ulcers, with consequent loss of gastric fluid.

Following sacrifice of the animals the esophagus was grossly examined for evidence of ulceration, the stomach was incised and its contents collected in a graduated centrifuge tube. The mucosal surface of the stomach removed from each fifteen-hour ligated rat was examined with the aid of a stereoscopic (wide-field binocular) microscope equipped with an eyepiece micrometer. The "degree of ulceration" was estimated by a modification of the characterization suggested by Keyrilainen and Passonen (6), an evaluation which takes into consideration both the number and size of the ulcers. This system is outlined in Table I.

TABLE I.—ULCER SCORING SYSTEM

Numerical Rating	Ulcer Score <sup>a</sup>	Descriptive Rating
0	0	Normal
1	Hemorrhagic	Irritation
2	Less than 4	Minimal ulceration
4	4 to 9	Moderate ulceration
6	10 to 19	Moderately severe ulceration
8	20 to 39	Severe ulceration
10	More than 39 or perforated	Very severe ulceration

<sup>a</sup> Ulcer score = total number of ulcers (gastric and esophageal) + total length of all ulcers in mm. (greatest dimension of each ulcer).

A study was undertaken to determine the relative activity of the seven anticholinergic compounds utilized in this investigation on the basis of reduction of the volume of gastric secretion. The intention was to establish "equipotent" antisecretory doses of these compounds in order that one of the factors being considered (i. e., volume of gastric secretion) would remain relatively stable throughout the investigation. The average volume of gastric fluid collected from four-hour pylorus-ligated rats following administration of several different doses of each of the seven anticholinergic drugs was compared to the average volume secreted by control animals which received an injection of a comparable volume of saline solution. All of the compounds utilized were dissolved in equal volumes of physiological saline and injected subcutaneously immediately after ligation of the pylorus. The slope of each dose-response curve indicated a linearity in response and no lack of parallelism among the various agents studied. The calculated equipotent doses (Table II), which were used in all subsequent studies, resulted in a volume of gastric secretion equivalent to 37–39% of the control volume. It should be noted that these doses may be considered "equipotent" only under the conditions of this investigation (i. e., four-hour pylorus-ligated rats of specified sex, weight, strain, etc.).

**Analytical Procedures.**—Acidity of the gastric samples was determined by titration with 0.02 *N* sodium hydroxide, using dimethylaminoazobenzene

TABLE II.—APPROXIMATE EQUIPOTENT DOSES OF ANTICHOLINERGIC DRUGS BASED ON VOLUME OF GASTRIC SECRETION IN FOUR-HOUR PYLORUS-LIGATED RATS

Compound	Dose, s. c., mg./Kg.	Number of Animals	Volume of Gastric Secretion, ml. $\pm$ S. D.
Control (saline)	...	122	5.85 $\pm$ 0.74
Atropine	10.0	30	2.22 $\pm$ 0.35
Oxyphenonium	1.0	30	2.25 $\pm$ 0.35
Tridihexethide	1.0	30	2.27 $\pm$ 0.48
Diphenmethanil	15.0	40	2.28 $\pm$ 0.36
Propantheline	0.05	40	2.31 $\pm$ 0.63
SCH 3085	2.0	30	2.23 $\pm$ 0.41
SCH 3444	1.0	40	2.29 $\pm$ 0.37

(Topfer's reagent) and phenolphthalein as indicators for free and total titratable acidity, respectively. Measurements of pH were made with a Beckman pH meter.

The method of proteolytic analysis employed in this study has been described previously (7). It represents an adaptation of Wesselman's (8) modification of the Anson and Mirsky (9) procedure, which essentially consists of quantitative proteolytic digestion of hemoglobin by pepsin. Results are expressed in milligrams equivalent to N. F. X Reference Pepsin.

Lysozyme activity was measured by a method previously described (10) which represents a modification of the bacteriolytic turbidometric procedures of Lobstein and Fogelson (11) and Smolelis and Hartsell (12). Employing crystalline lysozyme<sup>2</sup> from egg white as a provisional standard, results are obtained in micrograms of lysozyme.

Uropepsin determinations were performed by a method based upon the procedure of West, *et al.* (13). The technique basically consists of the precipitation of casein particles by a sample of activated urine containing the enzyme. Results were calculated according to the procedure of Hollander (14) and are expressed in units of uropepsin excreted per hour; one unit of activity being equivalent to 0.26 mcg. of crystallized pepsin (Armour) (15).

A colorimetric procedure for the determination of gastric mucin was adapted from the method of Glass, *et al.* (16, 17). Gastric samples were fractionated into three components, each with chromogenic activity. Gastric mucin is observed in both solid ("mueoid") and liquid ("mueoprotein" and "mueoproteose") fractions of gastric juice. Results are expressed as mg. of mucin in each fraction or in the entire sample.

## RESULTS AND DISCUSSION

**Four-Hour Pylorus-Ligated Rat Study.**—Determination of "equipotent" antisecretory doses in the four-hour pylorus-ligated rat indicated that, on a weight basis, considerable differences in antisecretory potency existed among the seven anticholinergic compounds evaluated (Table II). Measurement of several components of gastric secretion collected from pylorus-ligated rats four hours after administration of approximately equi-

<sup>2</sup> Kindly supplied by Armour Laboratories, Chicago, Ill.

potent antisecretory doses revealed a considerable specificity of action among the limited series of anticholinergic drugs employed in regard to the factors (gastric fluid volume, acidity, proteolytic activity, lysozyme activity, mucin content, and uropepsin activity) investigated.

In doses which uniformly reduced the volume of gastric fluid secreted during a four-hour period in pylorus-ligated rats to 37 to 39% of the control volume (i. e., approximate equipotent doses), all of the anticholinergic agents studied, with the exception of propantheline, significantly reduced proteolytic activity. Figure 1 indicates the mean proteolytic activity of gastric samples collected from anticholinergic treated rats in terms of per cent of the control value (i. e., data obtained from saline treated rats) on a concentration (mg./ml.) basis. It is evident that the per cent reduction in the total amount of enzyme present in the gastric secretion was considerably greater than that presented in Fig. 1, inasmuch as the volume of gastric fluid secreted during the experimental period was markedly inhibited.

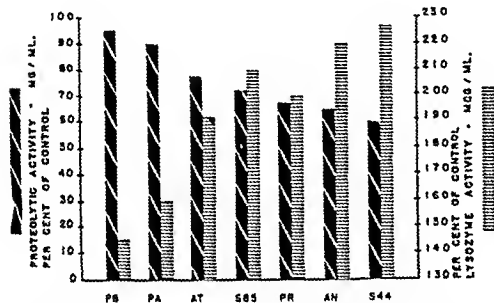


Fig. 1.—Proteolytic activity and lysozyme activity in the four-hour pylorus-ligated rat. PB, propantheline; PA, tridihexethide; AT, atropine; S85, SCH 3085; PR, diphenmethanil; AN, oxyphenonium; S44, SCH 3444.

The total lysozyme activity of the gastric juice collected from control rats ( $n = 30$ ) after a four-hour period of ligation was found to be equivalent to  $3.05 \pm 1.18$  mcg. of the enzyme. Administration of the anticholinergic compounds produced, in all cases, a significant reduction in the total content of lysozyme, but an elevation in the concentration of lysozyme in gastric fluid. These increases were in the range of 145–225% of normal levels of activity per ml. of gastric juice (Fig 1)

Gastric fluid obtained from four-hour pylorus-ligated rats which were not treated with anticholinergic drugs exhibited a pH of  $1.35 \pm 0.25$ . All seven anticholinergic compounds raised this value somewhat (average of pH 2.0), however, there were no obvious quantitative differences in this respect among the individual drugs. The anticholinergic drugs uniformly reduced the concentration of free gastric acidity (Fig 2) to approximately one-half of the control value, which corresponded to a decrease in free acidity in the entire gastric fluid volume to 15–20% of the control level.

None of the anticholinergic compounds significantly altered the uropepsin activity of urine col-

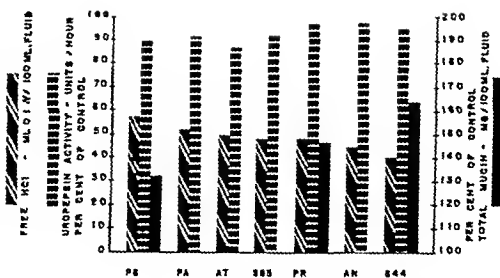


Fig. 2.—Free acidity, uropepsin activity, and total gastric mucin concentration in the four-hour pylorus-ligated rat. PB, propantheline; PA, tridihexethide; AT, atropine; S85, SCH 3085; PR, diphenmethanil; AN, oxyphenonium; S44, SCH 3444.

lected during the four-hour study (Fig. 2). Clinical reports are contradictory in regard to the effect of such compounds on uropepsin levels (18, 19). The amount of pepsinogen which enters the plasma appears to remain relatively constant, even after the administration of drugs which markedly reduce gastric pepsin activity. It would appear that none of the drugs, in the doses used, have a marked inhibitory effect on the endocrine activity of the chief cells; rather they appear to preferentially inhibit the exocrine function of these cells.

Of the three drugs studied for their effect on gastric mucin levels; propantheline, diphenmethanil, and SCH 3444; the latter compound produced the greatest increase (approximately 60% greater than the control level) in the concentration of gastric mucin (Fig. 2). However, a marked decrease was observed in the total amount of mucin secreted during the four-hour experimental period. This biphasic effect on concentration and total secretion of mucin, probably related to the inhibition of gastric fluid volume, was similar to that observed in regard to lysozyme activity. It has been suggested that both gastric mucoid and lysozyme are derived from the surface epithelial cells of the gastric mucosa, therefore it is conceivable that changes in the level of one of these components would be reflected by similar changes in the concentration of the other.

Within the series of anticholinergic drugs studied, a positive correlation was found to exist between the reduction of free acidity and the decrease in proteolytic activity of the gastric juice obtained from four-hour pylorus-ligated rats. An inverse relationship was observed between the effects of the anticholinergic agents on the proteolytic and lysozyme concentrations of gastric secretion. Lysozyme titer was found to be elevated to the greatest extent by those drugs which produced the greatest decreases in proteolytic activity. These effects appear to be partly attributable to the level of gastric acidity; a decrease in acidity being associated with a decrease in peptic activity (optimum pH 1.5–2.2) and a concomitant increase in lysozyme activity (optimum pH approximately 6).

**Fifteen-Hour Pylorus-Ligated Rat Study.**—All animals in this study were grouped into one of seven "ulcer classes" on the basis of ulcer score (Table I). Results of the analyses of the several components of gastric secretion (fluid volume, acidity, proteolytic activity, lysozyme activity, mucin content) were

evaluated on the basis of both drug treatment and severity of ulceration.

Doses of the seven anticholinergic drugs which produced approximately the same degree of inhibition of gastric secretion in the four-hour ligated rat were not "equipotent" in the fifteen-hour ligated animal, due largely to differences in duration of activity. Marked differences in the extent of protection provided against ulceration of these drugs were also noted (Table III). Although the lowest mean ulcer score was associated with the experimental group having the least gastric fluid volume and the highest ulcer scores were associated with groups evidencing the greatest volumes of gastric secretion, there was an inconsistent relationship among the remaining drug treated groups in regard to gastric fluid volume and severity of ulceration. However, if results are considered on the basis of ulcer response, without regard to drug treatment, there appears to be positive correlation between gastric fluid volume and the degree of ulceration produced by the experimental procedure employed in this study (Fig 3).

TABLE III.—INHIBITION OF GASTRIC SECRETION AND ULCERATION BY ANTICHOLINERGIC DRUGS IN THE FIFTEEN-HOUR PYLORUS-LIGATED RAT

Compound	Dose, s c , mg./Kg	n/Na	Volume of Gastric Secretion, ml $\pm$ S D.	Ulcer Score <sup>b</sup>
Control (saline)	...	25/30	13.57 $\pm$ 1.67	6.9
Atropine	10.0	10/10	10 53 $\pm$ 1.87	3.4
Oxyphenonium	1.0	9/10	9.24 $\pm$ 2.78	2.4
Tridihexethide	1.0	10/10	11.16 $\pm$ 1.89	2.9
Diphenmethanil	15.0	10/10	4.20 $\pm$ 1.03	0.0
Propantheline	0.05	10/10	10.21 $\pm$ 2.32	1.6
SCH 3085	2.0	9/10	13.23 $\pm$ 1.96	4.5
SCH 3444	1.0	10/10	8.58 $\pm$ 1.49	1.9

<sup>a</sup> Number of nonperforated animals/total number of animals employed. <sup>b</sup> Refer to Table I.

The effect of the anticholinergic agents on proteolytic activity was less evident in the fifteen-hour study than in the four-hour experiments. Only three compounds (tridihexethide, SCH 3085, and SCH 3444) significantly reduced proteolytic activity per unit volume of gastric secretion. Conceivably, the inhibitory effect of the anticholinergic drugs on peptic activity may terminate before their inhibitory influence on gastric fluid secretion. On the basis of grouped ulcer score, a definite correlation was demonstrated between the total proteolytic activity of the gastric fluid and severity of ulceration (Fig. 3). Again, the significantly greater fluid volume exhibited by the high ulcer score groups contributed markedly to this quantitative difference.

Considerable variation in the lysozyme concentration of gastric fluid was observed among the anticholinergic treated groups in the fifteen-hour study. In general, lower levels of lysozyme activity were found in ulcerated than in nonulcerated animals but no consistent relationship could be demonstrated between lysozyme concentration and severity of ulceration (Fig. 3).

The highest free acid concentrations were found in the secretions of those stomachs exhibiting

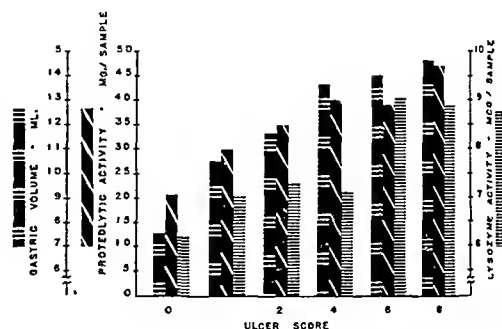


Fig. 3.—Comparison of gastric fluid volume, proteolytic activity, and lysozyme activity in the fifteen-hour pylorus-ligated rat on the basis of "ulcer score" (see Table I).

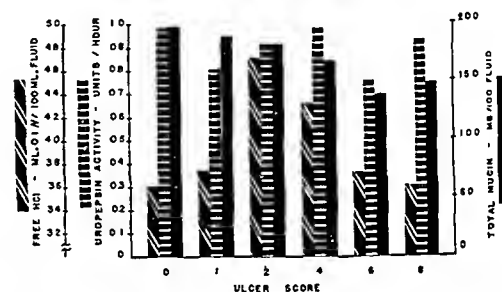


Fig. 4.—Comparison of free acidity, uropepsin activity, and total gastric mucin concentration in the fifteen-hour pylorus-ligated rat on the basis of "ulcer score" (see Table I).

moderate ulceration, whereas lower acid concentrations were associated with both lower and higher ulcer scores (Fig. 4). It is probable that neutralization of gastric acid by blood and tissue debris at the sites of ulceration may have been partly responsible for the reduced acid concentration observed in the severely ulcerated animals

As was noted in the results of the four-hour experiments, the seven anticholinergic drugs in the doses used, did not significantly alter the uropepsin activity of urine secreted during a fifteen-hour period of pylorus ligation. Furthermore, no relationship was observed between the degree of gastric ulceration produced by this technique and the levels of uropepsin activity (Fig. 4).

Prolongation of the period of pylorus-ligation from four to fifteen hours resulted in an average increase of 70% in the gastric mucin concentration of control (saline-treated) animals as contrasted to an average increase of only 25% in the anticholinergic treated groups. Since a large percentage of the increase in mucin concentration was due to elevated mucoprotein levels, it is not improbable that the increased amounts of gastric fluid associated with the extended period of ligation may have "washed out" preformed, but as yet nonsecreted, mucoprotein from the mucous neck cells. The highest mucin concentration was associated with the group evidencing minimal mucosal damage ("0" score). As the degree of ulceration increased, a decrease in mucin concentration was observed (Fig. 4). However, increases in the severity of

ulceration were accompanied by progressive increases in total mucin content of the gastric samples, apparently related to the higher average fluid volumes characteristically found in the more severely ulcerated animals.

## REFERENCES

- (1) Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Proc Soc Exptl Biol Med*, 65, 220 (1947)
- (2) Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Am J Med*, 5, 482 (1948)
- (3) Janowitz, H. D., Levy, M. H., and Hollander, F., *J Clin Invest*, 22, 670 (1951)
- (4) Gruenstein, M., *ibid*, 8, 627 (1947)
- (5) Gruenstein, M., and Passonen, M. K., *Acta Med Scand*, 173, 1 (1952)
- (6) Rossi, G. V., *Am J Pharm*, 130, 78 (1958)

- (7) Wesselman, H. J., *This Journal*, 45, 337 (1956)
- (8) Anson, M. L., and Mirsky, A. E., *J Gen Physiol*, 16, 59 (1932)
- (9) Goldberg, M. E., and Rossi, G. V., *Am J Pharm*, 130, 78 (1958)
- (10) Lobstein, O. E., and Fogelson, S. J., *Quart Bull Northwestern Univ Med School*, 25, 89 (1951)
- (11) Smolelis, A. N., and Hartsell, S. E., *J Bacteriol*, 58, 731 (1949)
- (12) West, P. M., Ellis, F. W., and Scott, B. L., *J Lab Clin Med*, 39, 159 (1952)
- (13) Hollander, F., *Gastroenterology*, 33, 659 (1957)
- (14) Rider, J. A., Moeller, H. C., Gibbs, J. O., Swader, J., Ageaoli, L. F., Deroin, J., Clark, I., and Anderegg, A., Scientific Exhibit, Booth 808, New York Meeting, American Medical Association, June 3, 1957
- (15) Glass, G. B. J., *Arch maladies App digest et maladies nutrition*, 20, 1017 (1938)
- (16) Glass, G. B. J., and Boyd, L. J., *Gastroenterology*, 12, 835 (1949)
- (17) Silver, H. M., Pucel, H., and Almy, T. P., *New Engl J Med*, 252, 520 (1955)
- (18) Corazza, L. J., and Myerson, R. M., *J Am Med Assoc*, 165, 146 (1957)

# Antithixotropic Behavior of Magnesia Magma\*

By CLIFFORD W. CHONG, STUART P. ERIKSEN, and JOSEPH V. SWINTOSKY

An unusual rheological behavior which involves a sol-gel transformation during measurement in a rotational viscometer, and a spontaneous return to a sol state on standing, has been observed in magnesia magma U. S. P. The phenomenon is the reverse of thixotropy. Thus, the term "antithixotropy" perhaps best describes this rheological behavior. This unique, seldom-encountered flow property was detected at shear rates of approximately 30 reciprocal seconds and greater, using a Drage rotating bob rheometer, and later confirmed with a modified Stormer viscometer. At rates of shear less than 30 reciprocal seconds, the material exhibited pseudoplastic flow. Rheograms of the data suggest that the increase in consistency during shear is due primarily to an increase in the apparent yield value with no significant change in the slopes of the flow curves. The result is a system which has good suspending characteristics, and one which, at the same time, is easily pourable. Equations analogous to those used for thixotropic and pseudoplastic flows are presented to describe the rate at which the resistance to flow increases with duration of shear, and the rate of decrease in the apparent viscosity with increasing rates of shear. A description of the Drage Viskostructure rheometer is also presented.

THIS PAPER reports the unique rheological behavior of magnesia magma U. S. P. Magnesia magma thickens when subjected to shear; on standing, it returns to a fluid state. Flow behavior of this type has been reported by other investigators (1-5) and has been designated "antithixotropy" (4) or "negative thixotropy" (2). Antithixotropy, heretofore found only in fluid polymer and pigment systems, has not been previously reported in pharmaceutical systems.

The purpose of this study was to characterize the antithixotropic behavior of magnesia magma in terms of parameters similar to those used to describe thixotropic and pseudoplastic flows

## EXPERIMENTAL

**Apparatus.**—The Drage Viskostructure rheometer and a modified Stormer viscometer were used in this study. The Drage rheometer, a fairly recent development, operates on the rotating bob-precision spring principle. It is capable of yielding data in fundamental units. The instrument consists essentially of a freely suspended synchronous motor, a calibrated precision spring, and four sets of measuring cups and bobs. The motor drives the measuring bob which hangs concentrically in the stationary measuring cup. The test fluid is placed in the annulus between the cup and bob, and exerts a frictional force when the bob is rotated. This force causes the motor to be twisted backwards against the calibrated precision spring until the resistance encountered by the bob is the same as the counterforce which results from the tension of the spring. The degrees through which the motor is turned are indicated on a dial, and are proportional to the fric-

\* Received August 21, 1959 from the Research and Development Division of Smith Kline and French Laboratories, Philadelphia 1, Pa.  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.



tional resistance exerted by the liquid on the bob. The instrument is capable of nine different speeds through the use of mechanical (gear ratio) and electrical (frequency modulation) changes. The rates of shear range from 4 to 1,600 reciprocal seconds, depending upon the cup and bob used.

The Stormer viscometer was modified only to the extent of making a new cup and bob. The cup had a diameter of 3.18 cm. and was 5.45 cm. in height, while the bob was 2.54 cm. in diameter and 3.90 cm. in height.

**Materials.**—Magnesia magma U. S. P. was studied. Samples prepared in our laboratory were made by (a) the U. S. P. X method<sup>1</sup> and (b) diluting a 30% Hydro-Magma<sup>2</sup> of magnesium hydroxide to 7.5% to conform to U. S. P. specifications. Commercial samples of magnesia magma, aluminum hydroxide gel U. S. P., and bismuth magma N. F. were also tested, the latter two for comparison only.

**Preparation and Prehandling of Test Samples.**—The test samples were prepared by placing the thoroughly mixed magma in round 120-ml. wide-mouth bottles. The bottles were capped and the samples were stored at room temperature for seven days. It was found that rheograms made daily showed no further change after the sixth day of storage, indicating that the magma had attained structural equilibrium in this time.

Immediately before measurement on the viscometer, each bottle of magma was placed tangentially on a horizontally rotating shaft (1-in. diameter), and turned end over end at 44 r. p. m. for exactly one minute. The magma was then transferred to the Drage measuring cup. The measuring bob was gently inserted, and the assembly placed in position on the Drage instrument, in a thermostatically controlled water bath set at  $25^{\circ} \pm 0.2^{\circ}$ . The viscometer was turned on after exactly fifteen minutes had elapsed from the time the magma was first agitated on the rotating mixer.

#### Rheological Measurements With the Drage Rheometer

**The Antithixotropic Rheogram.**—The general scheme for obtaining the data for the antithixotropic rheogram was to apply increasing rates of shear up to maximum value, followed by decreasing rates of shear. The time of shear at each shear rate was held constant while making both the up- and down-curves, i.e., the total time allowed for decreasing the rate of shear from the maximum shear rate was the same as the time used to increase it to this point.

**The Effect of Time at Constant Shear.**—Six separate samples were run in related manners, the only difference being the duration of shear at a constant initial rate of shear. The procedure is best visualized by reference to Fig. 2. The measurement of each sample was started at shear rate  $D_a$  which, in this case, was selected as the constant initial rate of shear. Sample 1 was sheared at rate  $D_a$  for fifteen seconds, then at rate  $D_b$  for fifteen seconds, and finally at rate  $D_c$  for fifteen seconds, yielding the shearing stresses  $a_1$ ,  $b_1$ , and  $c_1$ , respectively. Sample

2 was sheared an additional fifteen seconds at rate  $D_a$  or a total of thirty seconds, then at rates  $D_b$  and  $D_c$  in fifteen-second intervals. For each succeeding sample, the duration of shear at rate  $D_a$  was increased fifteen seconds beyond that of the previous sample before obtaining the shearing stress points of the upcurve at rates  $D_b$  and  $D_c$ .

Shear rates  $D_a$ ,  $D_b$ , and  $D_c$  shown in Fig. 2 corresponded to values of 344, 515, and 688 reciprocal seconds, respectively. Initial rates of shear ranging from 9.1 to 515 reciprocal seconds were employed in other measurements.

**The Effect of Shear at Constant Time.**—The up-curves of the rheograms for five magnesia magma samples were obtained as described above in "The Antithixotropic Rheogram." For each succeeding sample, the duration of shear at each shear rate was increased by fifteen seconds. Thus, the first curve was made using fifteen seconds at each shear rate, the second using thirty seconds, etc.

#### Rheological Measurements with the Stormer Viscometer

**The Antithixotropic Rheogram.**—In making the upcurve with the Stormer viscometer, the sample was first sheared with a net force of 26.4 Gm. and the time for 100 revolutions of the bob was recorded. A 2-Gm. weight was immediately added to the weight pan and the same sample was once more subjected to shear under this increased force. The procedure was repeated until, in addition to the 26.4 Gm., 14 Gm. had been added in 2-Gm. increments. Immediately after the final measurement of the upcurve, the procedure was reversed to form the downcurve, i.e., 2 Gm. was removed from the weight pan after each 100 revolutions of the bob. As a result, shear rate values were obtained at corresponding shearing stresses on both the up- and downcurves.

**The Effect of Time at Constant Shear.**—The sample was sheared with a net force of 25.4 Gm., and the time for 100 revolutions of the bob measured. The measurement was repeated several times using the same sample and the same shearing force until no further change in the time for 100 revolutions was noted.

## RESULTS AND DISCUSSION

The unique characteristic of magnesia magma is its reaction to shear. A pronounced increase in consistency may be induced by subjecting the material to a constant rate of shear greater than thirty reciprocal seconds. The degree of thickening depends upon the duration of shear, and, also, upon the magnitude of the stress applied. When sheared alternately with increasing followed by decreasing rates of shear, the material continues to thicken, but at a decreasing rate, until an equilibrium is reached (Fig. 1). At this stage the system is pseudoplastic, and is characterized by a high apparent yield value. Note, however, that the slope at any point along the pseudoplastic flow curve is practically the same as the slopes of the preceding upcurves at the corresponding rates of shear. On standing, the material spontaneously returns to a sol state characteristic of its structure prior to shearing.

Since the flow curves for magnesia magma were dependent upon both duration and rate of shear, it was desirable to use a standard procedure for pre-

<sup>1</sup> This method is also described in Remington's Practice of Pharmacy, 11th ed., Mack Publishing Co., Easton, Pa., p. 488.

<sup>2</sup> Produced by Merck and Co., and distributed by Whitaker, Clark, and Daniels, Inc., New York 13, N. Y.

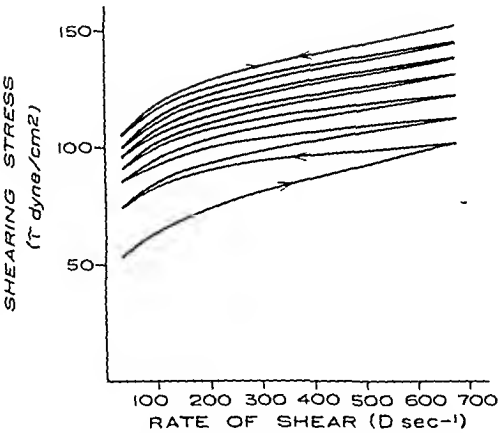


Fig. 1.—Rheograms showing the effect of repeated rheological measurements on magnesia magma.

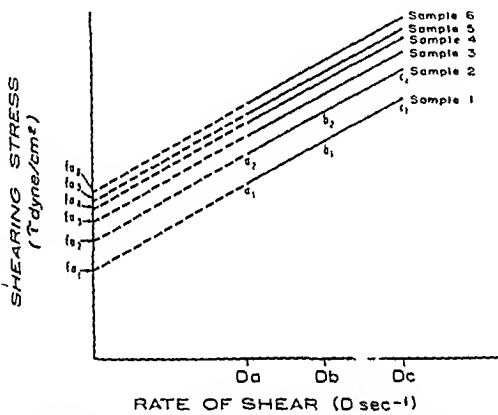


Fig. 2.—Rheograms showing the effect of duration of shear on magnesia magma at a constant rate of shear ( $D_a$ ).

paring and prehandling the test samples before the viscosity measurements were made. The data obtained by the proposed method were reproducible and comparable from one measurement to another. The magnesium hydroxide suspensions of this study gave rheograms of the type shown in the first cycle of Fig. 1. Several points of interest can be noted from an examination of the flow curve.

The upcurve is similar to the flow curve of a typical pseudoplastic substance. As the rate of shear is raised, the increase in resistance to shear,  $\tau(0)$ , per unit increase in the shear rate,  $D$ , approaches a constant value. Consequently, the upper portion of the curve approaches a straight line asymptotically. After the top rate of shear has been reached, the succeeding shearing stress measurements along the downcurve assume values larger than those on the upcurve at the corresponding rates of shear.

If, after the downcurve is completed, the rate of shear is promptly increased again, the points along the upcurve will be greater than those previously obtained at the same shear rates. The points along the subsequent downcurve will be even larger than those found earlier. Successive up- and downcurves

will yield larger and larger shearing stress values at each rate of shear. The rate of increase in shearing stress diminishes, however, until an equilibrium is established where both the up- and downcurves coincide as shown in Fig. 1. At this equilibrium the system is gel-like and possesses the unusual ability to suspend large, dense particles while still being readily pourable. For instance, glass beads, 1-mm. in diameter and having a density of 2.5 appear to remain suspended as long as the system is in this gel state. As the system spontaneously reforms its sol structure on standing, the glass beads slowly settle.

**The Effect of Time at Constant Shear.**—The effect that duration of shear has upon the resistance of magnesia magma to shear is shown in Fig. 2. The figure represents upcurves made on samples each of which had been subjected to increasing durations of shear at a constant initial shear rate indicated by  $D_a$ . The straight line portions of the upcurves, connecting the points  $a$ ,  $b$ , and  $c$ , show the parallel relationship between the slopes of the curves; also, extrapolation of the lines to the shearing stress axis indicates the apparent yield values,  $f_a$ . The qualification "apparent" is intended to indicate that the measured yield values vary with rate of shear.

A linear relation results when the shearing stress,  $\tau$  (or the apparent yield value,  $f_a$ ) is plotted against the logarithm of the time,  $t$ , at shear rate  $D_a$ . This is shown in Fig. 3. The relation between the change in shearing stress (or apparent yield value) and the duration of shear, then, may be expressed similarly to data of thixotropic flow systems (7).

$$R = \frac{\tau_2 - \tau_1}{\log t_2 / t_1} \quad \text{or} \quad = \frac{f_{a2} - f_{a1}}{\log t_2 / t_1}$$

where  $R$  is the constant which describes the interrelationship between shearing stress (or apparent yield value) and duration of shear, and might be termed the coefficient of increased resistance to shear with time. The magnitude of the constant  $R$  is a function of the constant rate of shear used,  $D_a$ , as indicated by the change in the slopes of the  $\tau$  versus  $\log t$  plots at various values of  $D_a$ .

It is interesting to note that the increase in shearing stress occurs only at rates of shear greater than

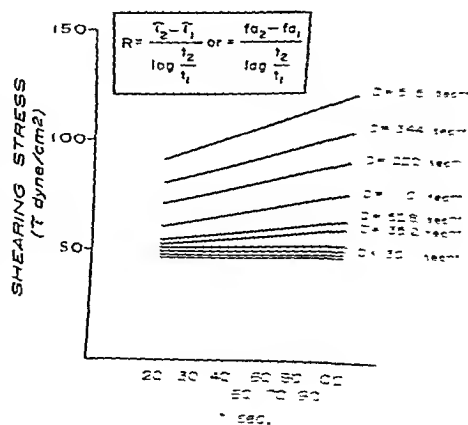


Fig. 3.—Increased resistance to shear of magnesia magma as a function of the rate of shear.

approximately thirty reciprocal seconds. Below this value, the effect of duration of shear is apparently nil. A rheogram of the data obtained at shear rates less than thirty reciprocal seconds results in a typical pseudoplastic flow curve as shown in Fig. 4.

In Fig. 5, values of  $\log R$  obtained above shear rates of thirty reciprocal seconds are plotted *versus*  $\log D$ . This plot yields a straight line which describes how the resistance to shear increases with time at various constant rates of shear.

**The Effect of Shear at Constant Time.**—Although there is an increase in the resistance to shear with time when magnesia magma is subjected to a constant rate of shear, there is, in addition, a decrease in the apparent viscosity with increasing rates of shear as illustrated in Fig. 6. Analogous to pseudoplastic systems (8), a straight line is obtained when the log of apparent viscosity,<sup>3</sup>  $\eta_a$ , is plotted against the log of shear rate,  $D$  (Fig. 7). The apparent viscosity in this case, is defined by the equation

$$\eta_a = \tau/D$$

which describes the slope of the line connecting the point  $(\tau, D)$ , on the upcurve of the rheogram, with the origin. The relation between the apparent viscosity and the rate of shear corresponding to it on the upcurve may be described by the equation

$$S = \frac{\log \eta_{a1}/\eta_{a2}}{\log D_2/D_1}$$

where  $S$  is the coefficient of decreasing apparent viscosity with increasing rates of shear. Figure 7 indicates that the constant  $S$  is relatively independent of the duration of shear at each shear rate.

It should be noted that the exact shape and position of the up- and downcurves, at constant durations of shear, will be governed in considerable measure by the instrument and the rates of shear chosen for the rheological measurements. The constants  $R$  and  $S$ , therefore, as determined in this study are not absolute values assignable to magnesia magma, but are rather relative values for determining the influence of (a) a constant rate of shear on shearing stress when the duration of shear is the variable, and (b) a variable rate of shear on shearing stress when the duration of shear at each shear rate is constant. By using this experimental approach, it has been possible to characterize the rheological behavior of magnesia magma, in a range of shear rates from nine to six hundred and eighty-eight reciprocal seconds, in terms of recognized rheological parameters.

**Studies with the Stormer Viscometer.**—It seemed desirable to use another viscometer to determine if the data obtainable from it would confirm the antithixotropic behavior of magnesia magma observed in studies with the Drage instrument. The Stormer viscometer was used for this purpose. Data which show the increased resistance to shear with duration of shear at a constant shearing force of 25.4 Gm. are given in Table I.

The rates of shear in Table I were calculated ac-

<sup>3</sup> Normally, for pseudoplastic systems the log of shearing stress,  $\tau$ , rather than the apparent viscosity,  $\eta_a$ , is plotted against the log of shearing rate,  $D$ , yielding a straight line of slope  $> 1$ . This plot yields a straight line for antithixotropic data also. Since, however, we desired to show the decrease in the ability of magnesia magma to resist flow with increasing rates of shear, it was felt that the apparent viscosity plot was more illustrative.

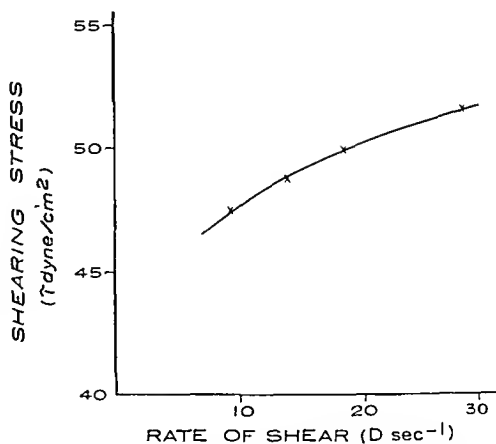


Fig. 4.—Rheogram of data obtained at rates of shear less than 30 sec.<sup>-1</sup>.

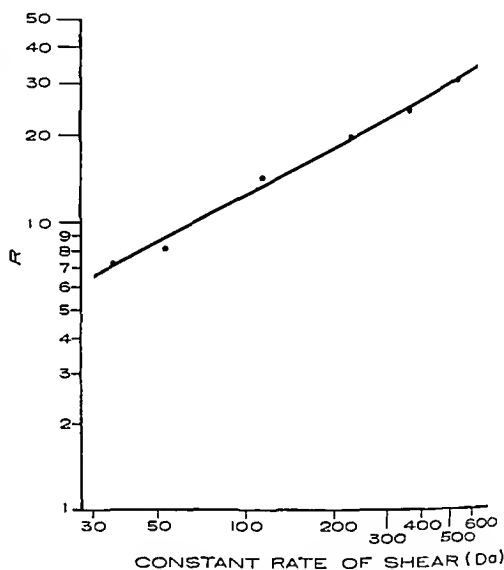


Fig. 5.—Plot of log constant rate of shear ( $Da$ ) vs.  $\log R$ .

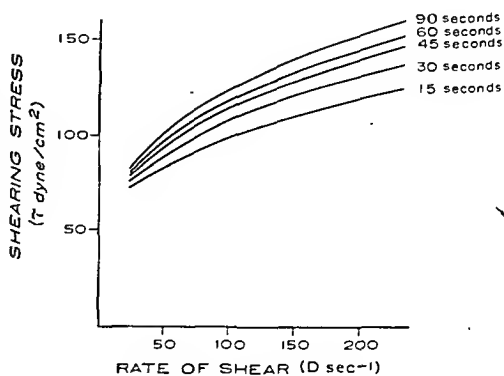


Fig. 6.—Rheograms showing the effect of duration of shear upon the upcurve.

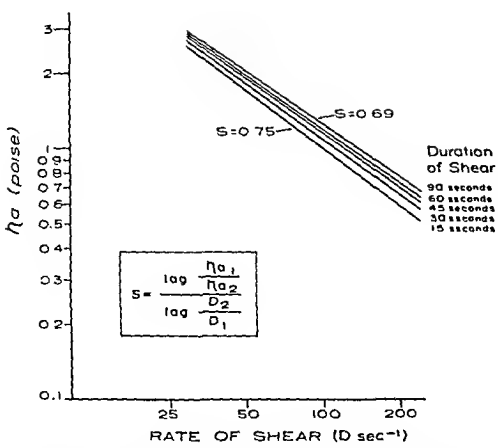


Fig. 7.—Plots of  $\log \eta_a$  vs.  $\log D$  showing the rates at which the apparent viscosity decreases with increasing rates of shear at various durations of shear

According to the method described by Reiner and Rivlin (9).

$$D = \frac{2 \text{ (r. p. m.) } R_c}{9.55 (R_c^2 - R_b^2)}$$

where  $R_c$  and  $R_b$  are the radii of the measuring cup and bob, respectively.

The gradual increase in the time of 100 revolutions of the bob indicates that magnesia magma increases in consistency when it is sheared repeatedly with a constant shearing force. After the seventh trial there is no further change in the time. This may be due to either or both of two things: (a) the system has attained structural equilibrium, or (b) since the rate of shear has decreased to less than thirty reciprocal seconds no further increase in consistency will occur.

Stormer viscometer data which show the antithixotropic behavior of magnesia magma are given in Table II. A rheogram of the data is shown in Fig. 8.

The Stormer viscometer operates only at selected shearing stresses. As a result, the duration of shear varies at each rate. Despite this inability to maintain a constant duration of shear, as one can with the Drage rheometer, the data obtained with the Stormer viscometer can be plotted to show the typical flow curves of time-shear dependent substances. Consequently, the Stormer rheogram in

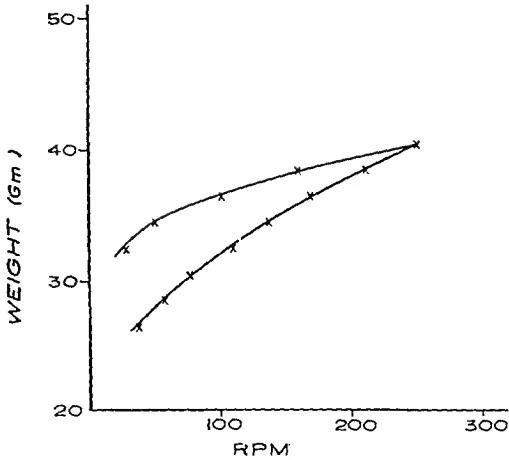


Fig. 8.—Stormer rheogram for magnesia magma.

Fig. 8 shows the characteristic antithixotropic flow curve as the Drage rheogram in the first cycle of Fig. 1.

The phenomenon of antithixotropy is seldom encountered in pharmaceutical systems. Even among the suspensions chemically related to magnesia magma, such as aluminum hydroxide gel U. S. P. and bismuth magma N. F., we failed to find signs of such behavior. Instead, these substances were thixotropic. A comparison of the flow curves of these substances with that of magnesia magma is shown in Fig. 9.

Although antithixotropy bears some resemblance to dilatancy and also to rheopexy, it nevertheless differs from them in several important respects. The rheological behavior of antithixotropic systems is dependent upon the duration as well as the rate of shear. Dilatant systems are dependent upon rate of shear only. Rheoplectic systems, i. e., those which gel more rapidly when a small shear is applied than when at complete rest (10), are gels at equilibrium; antithixotropic systems are sols.

Eliassaf, *et al.* (2), have suggested that antithixotropy in polymethacrylic acid solutions probably results from the fact that, during flow, the collision frequency of polymer molecules increases, causing a build-up of intermolecular bonds with time. Harvey, *et al.* (3), attributed this same rheological phenomenon in pigment suspensions to a shift in

TABLE I.—DATA OBTAINED WITH THE STORMER VISCOMETER SHOWING THE INCREASE IN THE RESISTANCE TO SHEAR WITH DURATION OF SHEAR AT A CONSTANT SHEARING FORCE OF 25.4 Gm.

Trial	1	2	3	4	5	6	7	8	9	10
Time of 100 rev. (sec.)	130	147	161	172	183	194	204	204	204	204
Rate of shear (sec. <sup>-1</sup> )	46.1	40.5	37.3	34.9	32.8	30.9	29.4	29.4	29.4	29.4

TABLE II.—DATA FROM STORMER VISCOMETER DEMONSTRATING ANTITHIXOTROPY

Driving weight, Gm.	26.4	28.4	30.4	32.4	34.4	36.4	38.4	40.4
Time of 100 rev., sec.								
Upcurve	158	106	76.8	54.4	43.6	35.6	28.4	24.0
Downcurve	...	...	750	214	119	59.6	37.6	...
Upcurve	38.0	56.6	78.1	110	138	169	211	250
Downcurve	...	...	8	28	50.4	101	160	...

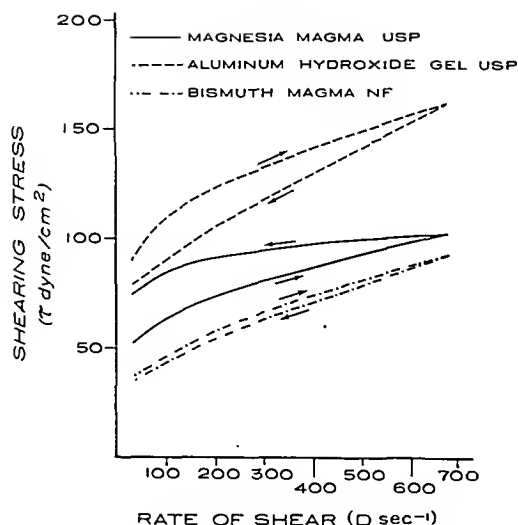


Fig. 9.—Drage rheograms for magnesia magma, aluminum hydroxide gel, and bismuth magma.

the equilibrium between two states of flocculates one having large numbers of small flocculates, and the other, small numbers of large flocculates. Both postulates probably describe similar phenomena, the second being the one most applicable to our system. From these theoretical conjectures, it would seem that the coefficient of increased resistance to shear should be some function of the rate of shear. Figure 5 indicates that with magnesia magma this is true. Also, it indicates that this coefficient is approximately equal to the square root of the shear rate used to obtain it (or is one-half order with respect to shear rate, in the kinetic sense).

Eliassaf also suggested that shear acts both in increasing and decreasing the resistance to shear. He attributed the difference between thixotropic and antithixotropic flows to a shift in the balance between these two effects. It appears from our data that a more complex situation exists in magnesia magma. At rates of shear below thirty reciprocal

seconds, the flow curve indicates pseudoplastic behavior. At shear rates greater than thirty reciprocal seconds, the particle collision frequency increases to such an extent that the rate of increased resistance to shear with time becomes noticeable; moreover, the rate of decreased apparent viscosity with increasing rates of shear is relatively insensitive to the duration of shear, as shown by the parallelism of the lines of Figs. 2 and 6. This suggests that the increased resistance to shear with time and the decreased apparent viscosity with increasing rates of shear are concerned with two separate and distinct features of the system.

We suggest that these features are the floccule size and their alignment. The apparent increased resistance to shear with time at a constant shear rate results from an increase of floccule collision and a consequent change in floccule size, the change progressing until an equilibrium state is reached. On the other hand, the decreased apparent viscosity with increasing rates of shear results from greater floccule alignment in the direction of flow. With alignment there is a lower energy requirement for the bob to move through the medium than would be necessary if the increased alignment had not occurred. Further study, however, is required to clarify this rheological behavior.

The possible applications of this type of rheological behavior to product development are receiving our consideration. Also, we have under preliminary investigation, several other systems which appear to exhibit antithixotropy.

## REFERENCES

- (1) Crane, J., and Schiffer, D., *J. Polymer Sci.*, **23**, 93 (1957).
- (2) Eliassaf, J., Silberberg, A., and Katchalsky, A., *Nature*, **176**, 1119 (1955).
- (3) Harvey, E. N., Bulas, R., and Fine, J., Paper Presented at the 124th Meeting of the Am. Chem. Soc., Sept. 6, 1953.
- (4) L'Hermite, *Ann. inst. tech. bâtiment et trav. publ.*, Sept., No. 92, No. 5, 1949.
- (5) Hartley, G. S., *Nature*, **142**, 151 (1938).
- (6) Williamson, R. V., *Ind. Eng. Chem.*, **21**, 1108 (1929).
- (7) Weltmann, R. N., *J. Appl. Physics*, **14**, 343 (1943).
- (8) Farrow, F. D., Lowe, G. M., and Neale, S. M., *J. Textile Inst.*, **19**, 18 (1928).
- (9) Reiner, M., and Rivlin, R., *Kolloid-Z.*, **43**, 1 (1927).
- (10) Freundlich, H., and Juliusburger, F., *Trans. Faraday Soc.*, **31**, 920 (1935).

# Synthesis and Metabolic Studies of C<sup>14</sup>-Labeled Hemicholinium Number Three\*

By FLOYD R. DOMER† and F. W. SCHUELER

Synthesis of C<sup>14</sup>-labeled hemicholinium No. 3 (HC-3) with a specific activity of 2.788 mc./mM is described. Twenty-four hour studies of rats in metabolism cages indicated 57.5 per cent of an injected dose is excreted in the urine, 15.6 per cent is excreted in the feces, and none is excreted in the expired carbon dioxide. Tissue distribution studies did not show any site of localization in rats.

IN 1954 LONG and Schueler (1) synthesized a series of compounds in an attempt to find a new type of anticholinesterase structure. Of special interest in this series was the compound which exhibited the lowest anticholinesterase potency. It proved to be the most toxic compound in causing death after a period of time had elapsed. A further study by Schueler (2) indicated that the compound, now called hemicholinium No. 3, (HC-3), depressed activity in the central nervous system. The potent respiratory depressant activity of this compound was attributed to its ability to form a hemiacetal configuration. Kasé and Borison (3) have shown HC-3 to have a primary site of action in the central nervous system.

HC-3 has also been found to have peripheral activity. In 1956 MacIntosh, *et al.* (4), showed that it had an effect on the rate of acetylcholine formation in the perfused superior cervical ganglion of the cat. Longo (5) recorded phrenic respiratory action potentials in rabbits and cats and obtained evidence compatible with neuromuscular blockage. Reitzel and Long (6) made an extensive study of the peripheral effects of HC-3 and found that it had neuromuscular blocking activity when the preparation being studied was stimulated fast enough to use up the stored acetylcholine. Wilson and Long (7) also found HC-3 had activity at numerous peripheral sites. It was shown to be much more potent as an inhibitor of transmission at the neuromuscular junction and parasympathetic end organs than

at a sympathetic ganglion and splanchnic nerve-adrenal gland junction. Desmedt (8) demonstrated that HC-3 caused results in skeletal muscle of cats which appeared to be identical with the characteristic picture seen in patients suffering from myasthenia gravis. Orkand and Martin (9) used intracellular electrodes in the frog foot to show that high doses of HC-3 prolonged the period of depolarization and changed the shape of the end plate potential. This indicates that HC-3 may have an effect directly on the muscle in sufficiently high doses.

The preceding discussion indicates the scope of the experiments which have been carried out to show the central and peripheral actions of HC-3. Numerous studies have indicated choline to be the most effective antagonist to the action of HC-3 (2, 6, 10-12). It has been suggested that HC-3 prevents the transport of choline to the necessary intracellular sites where it is acetylated to form acetylcholine. A possible site of HC-3 action which would account for its central nervous system activity is in the blood-brain barrier where it could prevent choline transport. To test this hypothesis the present studies involve the synthesis of radioactively-labeled HC-3, its localization in various body tissues, and routes of elimination from the body.

## EXPERIMENTAL

**Chemical Procedures.**—*Tertiary Amine Analog of HC-3.*—To 20 Gm. (71.6 mM) of *p,p'*-bisphenacyl chloride was added 6.45 Gm. (85.9 mM) N-methylaminoethanol in a flask placed in a water bath to prevent overheating. The reaction mixture was allowed to stand overnight. A 3-volume excess of water was then added and the reaction mixture allowed to stand overnight, during which time a brown precipitate formed. After washing several times with water the precipitate was collected on a filter. This product was extracted with boiling benzene and crystallized on cooling. It was repeatedly recrystallized from absolute ethanol. The melting point was 97–99° and the yield 58.4% of the theoretical amount. This agreed with the compound previously prepared in our laboratories by Dr. S. Kruger which was recrystallized from methanol. On analysis it was found to contain one mole of methanol of crystallization.

*Anal.*—Calcd. for C<sub>22</sub>H<sub>25</sub>N<sub>2</sub>O<sub>4</sub>·CH<sub>3</sub>OH: C, 66.34; H, 7.69; N, 6.73. Found: C, 66.27; H, 7.53; N, 6.68.

*C<sup>14</sup>-Hemicholinium No. 3.*—To 147.5 mg. (384

\* Received August 28, 1959, from Tulane University, School of Medicine, New Orleans 12, La.

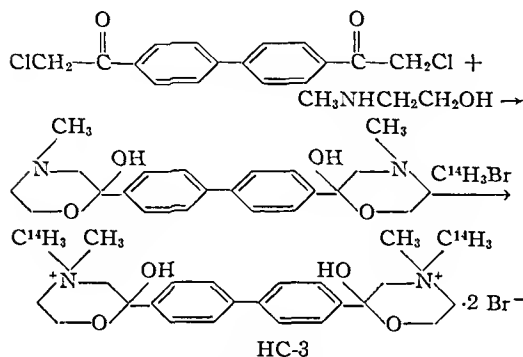
Supported by a grant No. CY 3037 (C2) from the U. S. Public Health Service.

Material drawn from a dissertation submitted to Tulane University in partial fulfillment for the degree, Doctor of Philosophy in Pharmacology.

† Present address: National Institute for Medical Research, Mill Hill, London, England.

The authors gratefully acknowledge the use of the facilities used in the Los Alamos Scientific Laboratories of the Atomic Energy Commission. Thanks also to Dr. Wright H. Langham and the members of the Biomedical Research Group who kindly supplied their time and information during the period of these experiments.

$\mu M$ ) of the tertiary amine thus prepared and placed in 8 ml. of 95% ethanol, 73 mg. of  $C^{14}H_3Br$  (753  $\mu M$ ) which had been prepared in the manner described by Foreman, *et al.* (13), was added. When the addition was complete the mixture was stirred at room temperature for twelve hours. An excess of nonradioactive  $CH_3Br$  (94.4 mg.; 994  $\mu M$ ) was then added and the reaction stirred at room temperature for forty-one hours. Precipitation was completed by adding absolute ether and cooling in a dry ice-acetone bath. The product was filtered on a tared, medium-fritted Büchner funnel. Further recrystallization from ethanol resulted in a 95.2% yield of the theoretical of the compound which melted at 178–180°. The reaction sequence is outlined below



The specific activity of the compound was determined by dissolving a sample of the HC-3 in a liquid scintillator solvent containing 2,5-diphenyloxazole (PPO) and 1,4-di-[2-(5-phenyloxazolyl)] benzene (POPOP) in toluene and counting on a Los Alamos liquid scintillation counter (14). The specific activity was 2.788 mc./mM. The radiochemical purity was found by autoradiography following descending paper chromatography. Whatman No. 1 paper 11½ × 18 in. was used with a solvent system of *n*-butanol-ethylene glycol-water (4:1:1 by volume). The chromatogram obtained in this procedure was placed against medical X-ray film and allowed to develop for an appropriate time. The  $R_f$  of the  $C^{14}HC-3$  in this system was 0.45.

**Biological Procedures.**—All animals used in these studies were male, Sprague-Dawley rats weighing approximately 250 Gm. The dose of HC-3 used was 40 meg./Kg. i. p. which is slightly less than the  $LD_{50}$  previously reported (2).

$C^{14}HC-3$  was given to four rats which were immediately placed in individual all-glass metabolism cages for twenty-four hours. The use of similar cages for mice has been previously described (15). They were so arranged as to collect separately the expired carbon dioxide in a sodium hydroxide solution, urine, and feces. An aliquot of the

sodium hydroxide solution was treated with an excess of a solution of 4 *M* ammonium chloride and 0.4 *M* barium chloride in  $CO_2$ -free water. The precipitated barium carbonate was collected on a medium-fritted Büchner funnel, dried in an oven, and weighed. A sample of this precipitate, along with aliquots of urine, feces, and other tissues were subjected to the procedure as has been previously described for counting of tissues in a refrigerated liquid scintillation counter (16).

A second series of animals were killed at varying periods of time after injection, i. e., twenty minutes, forty minutes, three, six, twelve, and twenty-four hours. One of each pair was then perfused via the left ventricle with about five blood-volumes of saline so as to remove the blood from the organs being studied. This is similar to the method of Schoolar, *et al.* (17). Tissues from both animals of the pair were then prepared and analyzed as in the previous section.

To study the possibility of secretion of the compound into the bile and on into the intestine, two chronic bile-fistula rats were prepared. The polyethylene cannula was run through a stab wound in the side of the animal. The animals were immobilized in Bollman-type cages and given 5% glucose in saline as drinking water and solid food, *ad libitum*. Following a forty-eight-hour recovery period  $C^{14}HC-3$  was injected and bile collected for two twelve-hour periods.

Autoradiographs were prepared of sections of the brain, liver, and kidneys from animals killed twenty minutes, forty minutes, and twenty-four hours after injection. Urine samples and water extracts of feces were chromatographed using the previously-mentioned *n*-butanol-ethylene glycol-water solvent system. These samples and chromatograms were then exposed to X-ray film for an appropriate length of time.

## RESULTS AND DISCUSSION

The results of the twenty-four hour metabolism experiments can be seen in Table I. It will be noted that animal No. 4 shows a wide discrepancy from the other animals. Presumably, this is due to the injection having been made into the intestine instead of into the peritoneal cavity. If this animal is neglected the average results show 57.5% is excreted in the urine, 15.6% excreted in the feces, and none excreted in the expired carbon dioxide. There was no radioactivity secreted into the bile in the chronic bile-fistula animals. This, coupled with the results seen in animal No. 4 in which the HC-3 was very poorly absorbed from the intestine suggests that the HC-3 is transported into the intestinal lumen from the surrounding peritoneal cavity or bloodstream while movement in the reverse direction is much slower. This would

TABLE I.—TWENTY-FOUR HOUR EXCRETION OF  $C^{14}$

Animal No.	Urine		Feces		$CO_2$	
	$C^{14}$ d. p. m. <sup>a</sup>	% Inj. Dose	$C^{14}$ d. p. m.	% Inj. Dose	$C^{14}$ d. p. m.	% Inj. Dose
1	75,184	56.7	14,212	10.7	557	0.42
2	54,252	45.4	26,914	22.5	0	0
3	95,309	70.3	18,298	13.5	0	0
4	3,390	3.0	90,292	81.9	0	0

<sup>a</sup> d. p. m. = Disintegrations per minute.

TABLE II.—C<sup>14</sup> TISSUE DISTRIBUTION OF TWENTY-MINUTE ANIMALS

Tissue	Unperfused			Perfused		
	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	2,628	2.4	223	0	0	0
Spleen	775	0.7	1,026	279	0.3	360
Kidneys	570	0.5	279	996	0.9	437
Muscle	0	0	0	0	0	0
Brain	420	0.4	225	183	0.2	103
Feces	3,799	3.5	2,282	2,880	2.7	1,734
Urine	546	0.5	910	0	0	0
Blood	57,312	53.1	2,274	18,631	17.2	736
Plasma	0	0	0	0	0	0

TABLE III.—C<sup>14</sup> TISSUE DISTRIBUTION OF FORTY-MINUTE ANIMALS

Tissue	Unperfused			Perfused		
	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	4,955	4.2	379	3,028	2.6	221
Spleen	1,046	0.9	1,187	468	0.4	521
Kidneys	3,526	3.0	1,575	1,761	1.5	733
Muscle	...	...	218	...	...	96
Brain	408	0.3	221	60	0.1	32
Feces	3,278	2.8	1,421	15,674	13.6	3,164
Urine	40,959	35.1	45,510	14,612	12.6	22,137
Blood	38,906	33.4	1,425	23,506	20.3	871
Plasma	0	0	0	0	0	0

TABLE IV.—C<sup>14</sup> TISSUE DISTRIBUTION OF THREE-HOUR ANIMALS

Tissue	Unperfused			Perfused		
	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	7,098	7.0	643	6,572	5.8	487
Spleen	959	0.9	1,391	1,833	1.6	1,660
Kidneys	2,567	2.5	1,307	1,856	1.6	714
Muscle	...	...	78	...	...	32
Brain	0	0	0	0	0	0
Feces	9,164	9.0	2,356	16,286	14.5	3,185
Urine	47,498	46.6	21,590	62,622	55.6	14,910
Blood	21,420	21.0	900	6,920	6.1	263
Plasma	0	0	0	0	0	0

TABLE V.—C<sup>14</sup> TISSUE DISTRIBUTION OF SIX-HOUR ANIMALS

Tissue	Unperfused			Perfused		
	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	2,694	3.0	316	7,550	7.6	626
Spleen	325	0.4	496	835	0.8	872
Kidneys	1,936	2.1	1,108	1,584	1.6	742
Muscle	...	...	179	...	...	378
Brain	261	0.3	158	0	0	0
Feces	19,154	21.0	5,839	6,885	7.0	4,167
Urine	58,734	64.4	32,630	71,743	72.6	19,390
Blood	7,950	8.7	373	22,755	23.0	985
Plasma	0	0	0	0	0	0

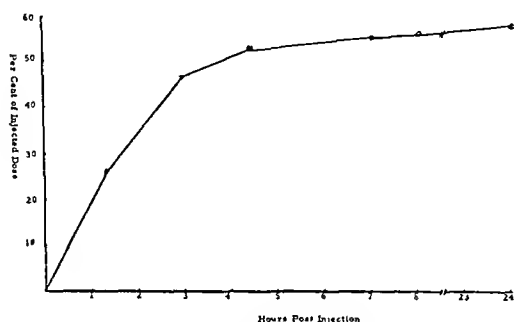
TABLE VI.—C<sup>14</sup> TISSUE DISTRIBUTION OF TWELVE-HOUR ANIMALS

Tissue	Unperfused			Perfused		
	d./min.	% of Inj. Dose	d./min./ Gm.	d./min.	% of Inj. Dose	d./min./ Gm.
Liver	6,757	6.1	701	2,217	2.1	189
Spleen	348	0.3	460	850	0.8	1,205
Kidneys	1,634	1.5	769	1,352	1.3	531
Muscle	0	0	0	0	0	0
Brain	0	0	0	0	0	0
Feces	30,287	27.2	5,747	26,819	25.9	10,737
Urine	78,498	70.5	12,460	63,423	61.2	23,490
Blood	25,379	22.8	976	24,424	23.6	1,009
Plasma	0	0	0	0	0	0



TABLE VII.—C<sup>14</sup> TISSUE DISTRIBUTION OF TWENTY-FOUR-HOUR ANIMALS

Tissue	Unperfused			Perfused		
	d./min.	% of Inj. Dose	d./min./Gm	d./min.	% of Inj. Dose	d./min./Gm.
Liver	3,015	2.7	369	1,260	1.1	110
Spleen	237	0.2	353	199	0.2	386
Kidneys	636	0.6	316	1,137	1.0	470
Muscle			203	0	0	0
Brain	0	0	0	0	0	0
Feces	90,292	79.6	17,321	26,914	22.5	3,782
Urine	3,390	3.0	295	54,252	45.4	3,487
Blood	0		0	18,930	15.9	678
Plasma	0	0	0	0	0	0

Fig. 1.—Typical urinary C<sup>14</sup> excretion following C<sup>14</sup> HC-3.

explain the previously-reported large increase in LD<sub>50</sub> when the compound is administered orally (2). Figure 1 shows typical results obtained with urinary excretion of the radioactivity. It indicates that the compound is very rapidly excreted into the urine. Autoradiographs of urinary chromatograms showed only one compound to be present. It had a *R<sub>f</sub>* of approximately 0.45 and was evidently the parent compound. This, along with the lack of respiratory C<sup>14</sup>O<sub>2</sub> suggests that there is little, if any, biotransformation of HC-3 in the body.

Tables II-VII show the tissue distributions at varying times after injection. It will be noted that in most of the twenty- and forty-minute animals some of the compound had already appeared in the urine and feces. This indicates the rapidity with which the body eliminates the HC-3. Table VI shows that 87-97% of the compound was eliminated in the urine and feces in the first twelve hours following injection. The only times at which any radioactivity was found in the brain were at twenty and forty minutes after injection (the times observed to be the onset of and most severe respiratory difficulty). Autoradiographs of the brain did not show any localization of the activity. This does not indicate that the blood-brain barrier is involved in the central activity of HC-3, but it also does not preclude the possibility that it may be acting there. An interesting point which can be seen in these data is that even though there was nearly always a detectable blood level of radioactivity none was ever found in the plasma. This suggests the HC-3 was adsorbed onto or held within the formed elements of the blood. When one considers the

acetylcholine-acetylcholinesterase activity of the erythrocytes this affinity is not too surprising. All of the sites of action which have thus far been found for HC-3 have been where cholinergic mechanisms exist. It will be noted that in some instances the return was greater than the injected amount of radioactivity. The most probable source of this error is the blood. In this analysis only 20% was used as an aliquot and the resulting solution was so highly colored there was a great deal of quenching. Both of these facts would contribute to error in the determination.

Autoradiographs of the kidney showed some localization of radioactivity in the pelvis and in the cortex and/or capsule. There was no localization of radioactivity in the liver.

## SUMMARY

The synthesis of C<sup>14</sup>-labeled hemicholinium No. 3 (HC-3) has been described. Twenty-four hour metabolism studies with rats indicated that the HC-3 is rapidly excreted in the urine and feces (ratio approximately 3:1). Tissue distribution studies in animals at various times after injection failed to show any sites of localization in various organs of the rat.

## REFERENCES

- (1) Long, J. P., and Schueler, F. W., *THIS JOURNAL*, 43, 79 (1954).
- (2) Schueler, F. W., *J. Pharm. Exptl. Therap.*, 115, 127 (1955).
- (3) Kasé, Y., and Borison, H. L., *ibid.*, 122, 215 (1958).
- (4) MacIntosh, F. C., Birks, R. I., and Sastry, P. B., *Nature*, 178, 1181 (1956).
- (5) Longo, V. G., *Arch. Intern. Pharmacodynamie*, 119, 1 (1959).
- (6) Reitzel, N. L., and Long, J. P., *ibid.*, 119, 20 (1959).
- (7) Wilson, B., and Long, J. P., *ibid.*, 120, 343 (1959).
- (8) Desmond, J. E., *Federation Proc.*, 18, 36 (1959).
- (9) Orkand, R. K., and Martin, A. R., *ibid.*, 18, 430 (1959).
- (10) Giovenco, J. F., *Bull. Fac. Tulane Med. School*, 16, 177 (1957).
- (11) MacIntosh, F. C., *Can. J. Biochem. Physiol.*, 37, 343 (1959).
- (12) Gardiner, J. E., *J. Physiol.*, 138, 13 P (1957).
- (13) Foreman, W. W., Murray, A. H., and Ronzio, A. R., *J. Org. Chem.*, 15, 119 (1950).
- (14) Hayes, F. N., *Intern. J. Appl. Radiation and Isotopes*, 1, 45 (1956).
- (15) Roth, L. J., Leifer, E., Hogness, J. R., and Langham, W. H., *J. Biol. Chem.*, 176, 249 (1948).
- (16) Domer, F. R., and Hayes, F. N., *Nucleonics*, 18, 190 (1960).
- (17) Schoolar, J., Barlow, J., and Roth, L., *Proc. Soc. Exptl. Biol. Med.*, 91, 347 (1956).

## Note on the Chemical Components of Extracts of *Eremocarpus setigerus*\*

By S. NAITO and C. R. NOLLER

NUMEROUS PLANTS were used by the native California Indians as fish poisons, but the two most highly esteemed for this purpose were *Chlorogalum pomeridianum* (soap root or amole), a member of the lily family, and *Eremocarpus setigerus* Benth (*Piscaria setigera* Piper; turkey mullein, or dove weed), a member of the spurge family (1). The active principles of the former are saponins which yield sugars and steroid sapogenins on hydrolysis (2).

The common names "dove weed" and "turkey mullein" refer to the fondness of wild turkeys and mourning doves for the seeds of the plant. "Mullein" refers to the woolly, mullein-like appearance of the leaves, which is due to prickly, compound stellate hairs that radiate outward from the leaf in all directions. Some Indians attributed the killing action on fish to these hairs, which, they said, attach themselves to the eyes and gills and make the fish frantic (3). However, it was a simple matter to show that a carefully filtered aqueous extract of the leaves and the unfiltered extract were equally toxic to goldfish. It is reported also (4) that the Indians applied the bruised fresh leaves to the chest as a counter-irritant for pain and that a weak decoction of the plant was used as a bath in typhoid and other fevers and taken internally as a cure for chills and fever. In our experience, concentrated extracts have a strong vesicant action, and collecting the plants for a whole day using leather work gloves eventually led to complete loss of the outer layer of skin of the palm and fingers of the right hand without the formation of blisters.

Preliminary examination, using goldfish for toxicity tests, showed that the active principle is extractable by acetone, methanol, ether, ethylene chloride, or benzene. In the general procedure used to obtain material for chemical investigation, the ground fresh plant, collected during the month of July, was extracted with hot methanol, and the methanol was removed under reduced pressure. The residue was a mixture of the water-soluble components dissolved in the water extracted from the plant, and the water-insoluble or slightly soluble components. Extraction of the mixture with benzene gave a benzene-soluble fraction and an aqueous solution. All of the active material was in the benzene extract and none in the aqueous layer. Accordingly the active principles are not water-soluble saponins.

Removal of the benzene from the extract left a dark green sticky residue. The residue was dissolved in peroxide-free ether and chromatographed

on alumina. Individual fractions were rechromatographed to give three crystalline products, which, in the order of decreasing ease of elution, proved to be paraffinic, alcoholic, and phytosterolic, none of which was toxic to fish. The melting points and analyses of these compounds agreed with those expected for hentriacontane, hexacosanol, and  $\beta$ -sitosterol, but the possibility that they are difficultly separable mixtures such as commonly are found in plant waxes (5) and plant sterol fractions (6) was not rigorously excluded. The more strongly adsorbed fractions were thick, sticky oils which were highly active. At a concentration of one part per million, the mean survival time of goldfish was seventy-five minutes for the crude benzene extract and twenty-two minutes after chromatographing. This toxicity may be compared with a survival time of forty-nine minutes for rotenone (7), two hundred minutes for croton oil, and thirty-five minutes for croton resin (8).

In another modification of the procedure, the residue left on evaporation of the benzene was distributed between hexane and aqueous methanol. All of the active material entered the methanol layer. Dilution of the methanol with water, extraction with ether, and evaporation of the ether left a sticky dark residue which was chromatographed on alumina. No crystalline fractions were obtained, and the toxic material appeared in the more strongly adsorbed fractions.

All attempts to obtain a single pure compound from the toxic fractions failed. Highly active fractions could be obtained by chromatography, but no crystalline product or crystalline derivative could be obtained. When a highly active fraction, separated by chromatography, was heated at 1.5 mm., a portion distilled in the range 130–185°. The portion boiling at 130–150° was inactive; that boiling at 160–185° was active, but not more so than the original fraction, and the portion distilling was small in amount.

Light-colored, highly-active fractions obtained by chromatography were free of nitrogen, sulfur, and halogen. Their infrared spectra indicated the presence of hydroxyl (2.98  $\mu$ , 3,355  $\text{cm}^{-1}$ ) and carbonyl groups (5.80  $\mu$ , 1,725  $\text{cm}^{-1}$ , and 5.85  $\mu$ , 1,710  $\text{cm}^{-1}$ ). However, all attempts to prepare crystalline derivatives using reagents for hydroxyl and carbonyl groups failed. Refluxing with alcoholic sodium hydroxide gave a neutral fraction and an acidic fraction. Both fractions were heavy sticky oils and no crystalline derivatives could be prepared from either fraction.

Because *Croton tiglium*, from the seeds of which is obtained the highly toxic croton oil, also is a member of the spurge family, the possibility of identity or similarity of our extracts to croton oil was con-

\* Received March 30, 1960, from the Department of Chemistry and Chemical Engineering, Stanford University, Stanford, Calif.

This investigation was supported in part by a PHS research grant, RG-5076, from the National Institutes of Health, U. S. Public Health Service.

sidered. The literature on the chemical nature of croton oil has been summarized recently (9). In contrast to croton oil, saponification did not yield a crystalline product analogous to "phorbol" (10), nor could any crystalline esters (9) be obtained. Boehm states (11) that croton resin gives an intense red-brown color with alcoholic potassium hydroxide or sodium ethoxide, a rose red and then dark brown color on boiling with alcoholic hydrochloric acid, and a grass green color when warmed with concentrated sulfuric acid. Active fractions from *Eremocarpus* are not changed in color by alcoholic sodium ethoxide, merely darken when boiled with alcoholic hydrochloric acid, and turn a dark reddish-brown when warmed with concentrated sulfuric acid.

## REFERENCES

- (1) Chestnut, V. K., *Contrib. U. S. Nat. Herbarium*, 7, 321(1902).
- (2) Noller, C. R., et al., *J. Am. Chem. Soc.*, 57, 525 (1935); 58, 1251(1936); 59, 1092(1937); 60, 1620, 1630 (1938); 61, 1707, 2420, 2976(1939); 63, 1240, 2131(1941); 70, 4260(1948).
- (3) Chestnut, V. K., *Contrib. U. S. Nat. Herbarium*, 7, 363(1902).
- (4) *Ibid.*, 7, 364(1902).
- (5) Chibnall, A. C., Piper, S. H., Pollard, A., Williams, E. F., and Sahai, P. N., *Biochem. J.*, 28, 2189(1934).
- (6) Karrer, W., "Konstitution und Vorkommen der Organischen Pflanzenstoffe," Birkhaeuser Verlag, Basel Switzerland, 1958, p. 845.
- (7) Gersdorff, W. A., *J. Am. Chem. Soc.*, 52, 3440(1930).
- (8) Spies, J. R., *ibid.*, 57, 180(1935).
- (9) Thomas, A. F., and Marxer, A., *Experientia*, 14, 320 (1958).
- (10) Flaschentraeger, B., *Ber. ges. Physiol. u. expil. Pharmacol.*, 42, 585(1928).
- (11) Boehm, R., *Arch. expil. Pathol. Pharmacol.*, 79, 135 (1915).

## Note on the Characteristics of an Apomorphine Response in Pigeons\*

By A. M. BURKMAN

Quantitative relationships between the dose and incidence, latent period, duration of action, cumulative and minute responses of the apomorphine-induced pecking syndrome are described.

APOMORPHINE has long been recognized as a potent emetic in man and other animals. This substance shares with its parent morphine the property of stimulating the chemoreceptor trigger zone (CTZ) of the medulla thereby activating the vomiting center (1). CTZ excitation represents one of perhaps several nervous system stimulating components of apomorphine's spectrum of activity. The drug's emetic propensity is most readily exhibited in man and the dog, two animals who have proved to be exquisitely sensitive to this drug. Without doubt, emesis is the most apparent sign of apomorphine-induced central stimulation (following the administration of comparatively low doses) in these animals.

One sees evidence of apomorphine stimulation in a number of other animal species particularly those that are refractory to the emetic effect. In the frog, apomorphine is responsible for a marked increase in reflex excitability (2), while restlessness, excitement, and a peculiar gnawing are characteristic effects in rabbits (3). In a somewhat similar manner, the pig, rat, horse, and guinea pig also display distinct signs of stimulation (4).

The excitatory action of apomorphine manifests itself in pigeons in a manner strikingly different from that described for other animals. Following

administration by any one of several routes (i. v., i. p., i. m., p. o.) this bird exhibits what has been described to be a "pecking syndrome" (5) or "feeding hallucination" (6); a continuous and repetitious pecking at the floor, walls, and roof of the cage in which it has been placed.

The relationships between the dose and a number of characteristics of this curious pecking response are herein reported.

Adult domestic pigeons of both sexes served as test subjects. All apomorphine hydrochloride solutions were administered by the intraperitoneal route, and the bird's responses monitored continuously for four hours following injections. The following observations can be reported on the basis of data contained in Figs. 1 and 2:

1. The  $ED_{50}$  (and 95% confidence limits) of apomorphine HCl, determined by the method of Litchfield and Wilcoxon (7), was found to be 0.154 (0.134-0.177) mg./Kg. while the slope function of the dose-response line (reproduced in Fig. 1) was calculated to be 1.54 (1.21-1.95).

2. Latent period following injection was observed to decrease as the dose increased. The regression line formula was calculated to be  $Y = 7.39 - 2.65 X$  (Fig. 2 A).

3. Pecking activity (pecks/minute) is stimulated to the greatest degree when doses of about 1.0 mg./Kg. (log dose 0) are reached (Fig. 2 B). Doses beyond this maximum effective dose level failed to increase the minute activity, this no doubt due to physiological limitations of the act.

4. The duration of action is an almost linear function of the log dose throughout the investigated range (Fig. 2 C).

5. The cumulative (total) response, as one might

\* Received November 13, 1959, from the College of Pharmacy, University of Illinois, Chicago.

This project was supported by funds made available by the Research Board of the University of Illinois at the Chicago Professional Colleges.

August 1960

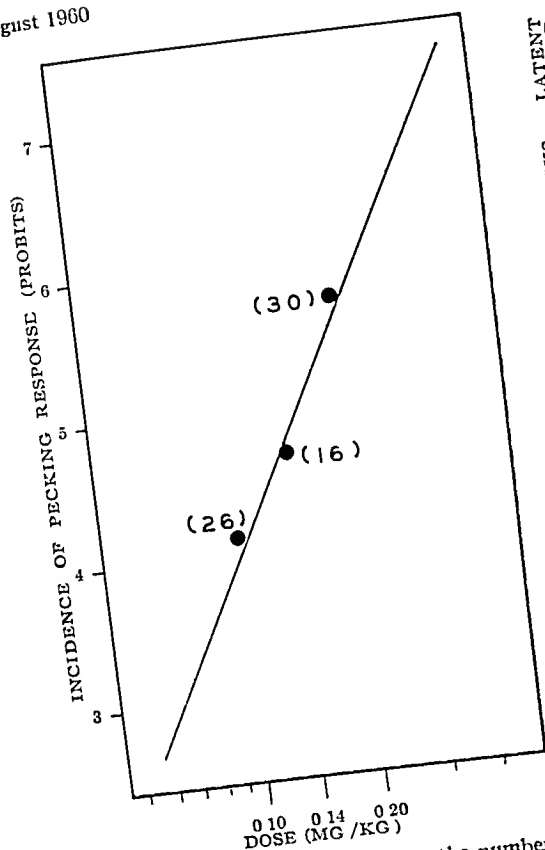


Fig 1.—The relationship between the number of pigeons exhibiting a positive pecking response and the dose of apomorphine HCl administered intraperitoneally. Numerals in parentheses represent the total number of birds used to plot the respective points

expect after examining the above data, also increases with increase in dose. Beyond the maximum effective dose the increase in total pecking would be a direct result of increasing duration. The leveling off of the cumulative response-log dose curve (Fig 2 D) at high doses warrants explanation inasmuch as the duration curve maintains a nearly continuous slope. It is suggested that this leveling off may only be an apparent statistical effect particularly since the plotted point at log dose 2 (in Fig 2 D) has a rather large standard error.

Doses up to and including 100 mg /Kg gave rise to no deleterious effects in the pigeon. Five out of

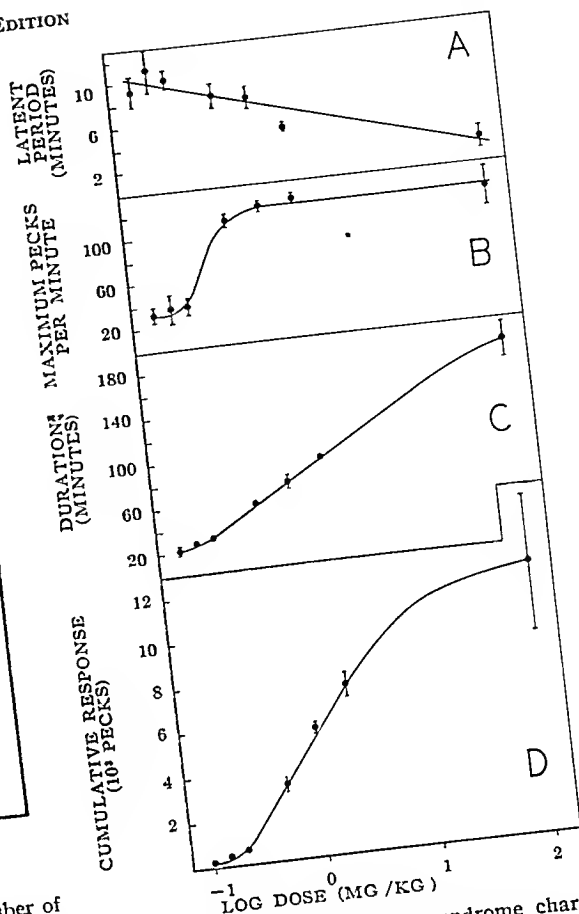


Fig 2.—Relation of pecking syndrome characteristics to dose. Each point represents the mean ( $\pm$  standard error) of 12–25 pigeons.

a total of 66 animals exhibited from one to four brief vomiting episodes each lasting fifteen seconds or less. No response other than repetitive pecking was observed in the other pigeons.

#### REFERENCES

- (1) Wang, S C, and Borison, H L, *Gastroenterology* 22, 1(1952)
- (2) Hattori, T, *Arch intern Pharmacodynamie*, 20, 57 (1910)
- (3) Harnack, E, *Arch expil Pathol. Pharmacol Naunyn-Schmiedeberg's*, 2, 254(1874)
- (4) Krueger, H, Eddy, N, and Sumwalt, M, *Public Health Repts U S*, Suppl 165, pl 2, 921(1943)
- (5) Burkman, A M, Tye, A, and Nelson, J W, *THIS JOURNAL*, 46, 140(1957)
- (6) Koster, R, *J Pharmacol Exptl Therap*, 119, 406 (1957)
- (7) Litchfield, J T, and Wilcoxon, F, *ibid*, 96, 99(1949)

# Note on Suitable Solvent Systems Usable in the Liquid Scintillation Counting of Animal Tissue\*

By GERALD A. BRUNO and JOHN E. CHRISTIAN

Animal tissues were dissolved, using hyamine base as the solvent, by several different techniques. Tissues exposed to ultrasonic excitation or homogenization upon application of heat dissolved sooner than untreated tissue. Addition of a known amount of carbon-14 standard to the tissue samples and counting of the samples by liquid scintillation techniques indicated that the methods employed for tissue solution yield satisfactory solutions for routine liquid scintillation analysis of carbon-14 in animal tissue.

WITH THE ADVENT of liquid scintillation counting in 1950, a technique especially applicable to the counting of weak beta-emitting isotopes was realized. Using the weak beta-emitting isotopes, carbon-14 and tritium (hydrogen-3), an unlimited number of compounds can be labeled and assayed with a high degree of accuracy.

This technique is particularly important to the investigator who wishes to determine the distribution and metabolism of various compounds in the animal body. With this application arises the difficulty of preparing suitable scintillator solutions of the various constituents of the animal body. The primary purpose of this investigation was to study suitable scintillator solvents for animal tissues. The instrument used was the Tri-Carb liquid scintillation spectrometer.<sup>1</sup>

The problem of simple preparation of animal tissue containing carbon-14 or tritium for scintillation counting, has been approached in several ways with varying degrees of effectiveness. Indirect methods reported, consist of grinding and suspending the insoluble tissue in a scintillation solvent such as toluene (1-4). The search for a simpler, more direct method of preparing clear scintillator solutions of animal tissue led to the discovery that a methanolic solution of the hydroxide form of a quaternary amine could be used to dissolve animal tissue (5). The quaternary amine chloride, commonly known as Hyamine 10X<sup>2</sup> (methylbenzethonium chloride) is commercially available but must undergo a relatively lengthy purification and conversion process before it can be used as a satisfactory solvent. The purified form of the Hyamine chloride is converted to the free hyamine base (methylbenzethonium hydroxide) by treatment with silver oxide (6). It has been reported that approximately 100 mg. of protein can be dissolved in 1-3 ml. of the Hyamine base (5). Since this report, several articles have appeared suggesting methods for improving the preparation procedure (7) and for modifying the method of dissolving the tissue in Hyamine (8, 9). However, no information has been reported as to the relative efficiency of detection for C<sup>14</sup> in the tissue systems reported.

## EXPERIMENTAL

The optimum window for the Tri-Carb was determined to be 6-90 volts and was used in all subsequent experimentation.

The use of silver oxide as described by Passman, *et al.* (6), for conversion of the purified chloride to the base, yielded a colored solution of the base. Experimentation in this laboratory revealed that methanolic sodium hydroxide could be used to convert the chloride to the base without the formation of the highly undesirable color. A 47.8-Gm. portion of the purified Hyamine chloride was dissolved in 50 ml. of methanol and treated with 50 ml. of an 8% methanolic sodium hydroxide solution. The reaction mixture was cooled to precipitate as much sodium chloride as possible and filtered. A clear, colorless, approximately 1.0 M solution of the base in methanol was obtained.

Preliminary investigation using the 1.0 methanolic Hyamine base solution to dissolve rat tissue with direct heat yielded colored solutions and generally poor counting conditions. This undesirable color resulted from prolonged heating found to be necessary to solubilize the tissue samples, and the extent of the color was found to be related to the particular type of tissue being dissolved. In an effort to avoid prolonged heating, the only factor in color production which can be controlled at present, ultrasonic waves as a tissue solubilizer and homogenization procedures were employed. The accurately measured tissue samples, weighing approximately 75 mg., were placed directly in the counting vials<sup>3</sup> with 2 ml. of Hyamine base solution added to each. The homogenization was accomplished by breaking up the tissue with a glass stirring rod, since homogenization by other means with such small quantities was impractical. The ultrasonic wave treatment was accomplished by placing the vial containing the tissue suspended in 2 ml. of the Hyamine base solution in the transducer<sup>4</sup> of an ultrasonic generator and insonating it for one hour at 400 kc. Each of the samples, including an untreated sample, were heated in an oven at 100° until solution of the tissue was complete. The time for solution was noted in each instance.

The vials containing the dissolved tissue were cooled and diluted with 13 ml. of scintillator solution consisting of 4 Gm./L. of PPO and 0.1 Gm./L. of POPOP in toluene. A 20% solution of concentrated sulfuric acid in absolute ethanol was used to acidify each solution as indicated by litmus paper. This procedure eliminated the possibility of protein phosphorescence in alkaline media and thus assured the elimination of spurious counts. A known and identical amount of C<sup>14</sup> standard<sup>5</sup> was added to each vial and the activity determined at the tap

\* Received April 4, 1960, from the Bionucleonics Dept., Purdue University, Lafayette, Ind.

<sup>1</sup> Model 314X, Packard Instrument Co., Inc., LaGrange, Ill.

<sup>2</sup> Rohm and Haas Co., Philadelphia, Pa.

<sup>3</sup> Special low-potassium 20-ml. vials with tin foil-lined screw cap, Wheaton Glass Co., Millville, N. J.

<sup>4</sup> Hypersonic Transducer, model Bu 301, frequency 400 kc., Brush Development Co., Cleveland, Ohio.

<sup>5</sup> National Bureau of Standards, beta-ray standard, carbon-14, benzoic acid in toluene, 16,500 dpm. per Gm. of solution.

setting yielding the highest count rate for each solution. The results listed in Table I indicate that the use of ultrasonics as a tissue solubilizer was advantageous, as was homogenization. In general, the heating time of the insonated tissue was less than that required by the other two methods. The results also indicate that all the tissues tested can be assayed for  $C^{14}$  with satisfactory accuracy; however, the absolute efficiency varies with the tissue involved, emphasizing the need for internal standard methods of comparison. The internal standard method makes use of the fact that upon addition of a known amount of activity one can then calculate the degree of quenching of the solution in question, thereby giving a measure of absolute activity.

TABLE I.—COMPARISON OF TIMES REQUIRED FOR SOLUTION AND CARBON-14 ABSOLUTE EFFICIENCIES OF SOLUTIONS OF VARIOUS TISSUES DISSOLVED IN HYAMINE AT 100° AND COUNTED IN THE TRI-CARB LIQUID SCINTILLATION SPECTROMETER

Tissue		Time, <sup>a</sup> min.	High Voltage, <sup>b</sup> Tap	Absolute Efficiency, <sup>c</sup> %
Brain	(a) <sup>d</sup>	25	8	36
	(b)	12	6	56
	(c)	15	6	68
Liver	(a)	60	9	24
	(b)	30	7	41
	(c)	5	7	45
Kidney	(a)	120	7	36
	(b)	15	6	59
	(c)	15	6	58
Muscle	(a)	45	6	59
	(b)	25	6	63
	(c)	20	6	61
Heart	(a)	30	9	23
	(b)	20	7	43
	(c)	20	7	54
Spleen	(a)	45	9	18
	(b)	30	7	37
	(c)	30	8	28
Intestine	(a)	60	9	42
	(b)	45	7	55
	(c)	35	7	53
Standard <sup>e</sup>	..	..	5	73

<sup>a</sup> Time in minutes required to completely dissolve tissue at the arbitrarily chosen 100° heating temperature

<sup>b</sup> Tap setting which gives maximum count rate

<sup>c</sup> Absolute efficiency is determined by dividing the count rate of the tissue solution by the absolute disintegration rate of the known amount of standard added to each sample

<sup>d</sup> (a) Direct heating of intact tissue, (b) direct heating of homogenized tissue, (c) direct heating of tissue exposed to ultrasonics

<sup>e</sup> Solution consists of NBS carbon-14 standard in scintillator solution of 4 Gm./L. of PPO and 0.1 Gm./L. of POPOP in toluene.

## DISCUSSION

It is important to note that results obtained for  $C^{14}$  are not wholly applicable to tritium determinations. Tissue solutions which counted with an absolute efficiency of over 50% could likely be assayed for tritium with relatively high efficiency, but the less efficient solutions which were counted at high voltage tap settings of 8 and 9, would probably be too highly quenched to be of any value in tritium counting.

To obtain an efficient scintillator solution for animal tissue, the important factors to be considered are (a) use of a solvent directly miscible with the organic scintillator, to minimize adulteration of the solution with solubilizing additives, and (b) use of whatever auxiliary means available to obtain a clear, colorless, unquenched solution.

The color produced by heating of the Hyamine base could possibly be reduced by determining the optimum temperature for tissue solution with minimum color production.

## SUMMARY

1 Hyamine base was prepared by conversion of purified Hyamine chloride to the free base by treatment with methanolic sodium hydroxide. This procedure produced Hyamine base without the objectionable color previously encountered.

2 Various tissues were dissolved in Hyamine using (a) direct heat only, (b) homogenization and heat, and (c) ultrasonic excitation and heat. In general the tissue exposed to ultrasonic waves dissolved sooner than the untreated tissue, as did the homogenized tissue, but to a lesser extent. Reduction of heating time brought about a marked decrease in the color intensity of the samples.

3 The samples with little or no color produced during heating gave considerably higher absolute counting efficiencies than untreated tissue samples which produced highly colored solutions. Absolute efficiencies ran as high as 68% and in each instance were of sufficient magnitude to permit routine analyses.

## REFERENCES

- (1) Hayes, F. N., Rogers, B. S., and Langham, W. H. *Nucleonics*, 14, 48 (1956)
- (2) Funt, B. L., *ibid.*, 14, 83 (1956)
- (3) Funt, B. L., *Sci.*, 125, 986 (1957)
- (4) White, C. G., and Helf, S., *Nucleonics*, 14, 46 (1956)
- (5) Vaughan, M., Stenberg, D., and Logan, J., *Sci.*, 126, 446 (1957)
- (6) Passman, J. M., Radin, N. S., and Cooper, J. A. D., *Anal. Chem.*, 28, 484 (1956)
- (7) Bell, C. G., and Hayes, F. N., "Liquid Scintillation Counting," Pergamon Press, New York, N. Y., 1958, p. 123
- (8) Herberg, R., *Sci.*, 128, 99 (1958)
- (9) Bell, C. G., and Hayes, F. N., "Liquid Scintillation Counting," Pergamon Press, New York, N. Y., 1958, p. 228

# Note on the Constituents of the Indian Medicinal Plant *Oldenlandia corymbosa* Linn.\*

By H. N. KHASTGIR, S. K. SENGUPTA, and P. SENGUPTA

$\gamma$ -Sitosterol and the triterpene acids, oleanolic acid and ursolic acid, have been shown to be present in the Indian medicinal plant *Oldenlandia corymbosa* Linn. Evidence is presented to show that the plant does not contain any alkaloid.

**O**LDENLANDIA CORYMBOSA Linn. (Sanskrit: Par pata) of N. O. Rubiaceae is a common plant growing throughout India. The plant is used in remittent fever with gastric irritation and in nervous depression (1). A study of the plant appeared desirable because of its medicinal importance and because no chemical investigation of it had been reported in the literature. The only members of *Oldenlandia* family that have been investigated chemically are *O. auricularia*, *O. biflora*, and *O. herbacea*. New alkaloids were reported to have been isolated from *O. auricularia* (2) and *O. biflora* (3). Later Govindachari and co-workers (4) showed that a genuine sample of *O. biflora* did not contain any alkaloid. This group of workers isolated ursolic acid from *O. biflora* and *O. herbacea*. More recently Bhakuni (5) isolated  $\beta$ -sitosterol and  $\gamma$ -sitosterol from the seeds of *O. biflora*.

In the present study the dried plant of *O. corymbosa* was defatted with petroleum ether. From the unsaponifiable fraction of the fat  $\gamma$ -sitosterol was isolated and identified by the preparation of its acetate and benzoate derivatives. The defatted plant was then extracted with benzene. The residue from the benzene extract on crystallization from acetone afforded crude ursolic acid, which was purified and identified as its methyl ester and methyl ester acetate. The acetone mother liquor yielded a solid residue, which on acetylation followed by crystallization afforded oleanolic acid acetate, which in turn was further identified by the preparation of its methyl ester acetate.

The plant was finally extracted with 95% ethanol, but no crystalline material could be isolated from this fraction. Each of the petroleum ether, benzene, and ethanolic extracts gave negative tests for alkaloids.

## EXPERIMENTAL

### Extraction with Petroleum Ether and Isolation of the Unsaponifiable Material

Dried and crushed whole plant (2.2 Kg.) of *O. corymbosa* was extracted in a Soxhlet apparatus with petroleum ether, b. p. 60–80° for two hours. The petroleum ether was distilled off and the residual fat (20 Gm.), which showed negative test for alkaloids, was saponified by refluxing for two hours with a solution of potassium hydroxide (10 Gm.) in methanol (30 cc.) and water (2 cc.). The reaction mixture was diluted with water and the unsaponifiable material was extracted with ether, washed with water, and dried over anhy-

drous sodium sulfate. The removal of ether yielded the unsaponifiable material (3.5 Gm.), which was chromatographed over activated alumina (100 Gm.). Elution with ether gave a partially crystalline material (0.82 Gm.).

$\gamma$ -Sitosterol.—The partially crystalline material (0.82 Gm.) obtained above was rechromatographed over activated alumina (50 Gm.), again eluted with ether, and the resulting crystalline solid (0.38 Gm.), was recrystallized from methanol to yield  $\gamma$ -sitosterol, m. p. 146–148°,  $[\alpha]_D - 42.4^\circ$  (CHCl<sub>3</sub>). [Reported (6), m. p. 147–148°,  $[\alpha]_D - 43^\circ$ .] The sample showed a positive Liebermann test.

$\gamma$ -Sitosterol Acetate.—The acetate was prepared in the usual manner with acetic anhydride and pyridine. On crystallization from methanol and then from acetone, it gave  $\gamma$ -sitosterol acetate, m. p. 142–143°,  $[\alpha]_D - 46^\circ$  (CHCl<sub>3</sub>). [Reported (6), m. p. 143–144°,  $[\alpha]_D - 45.3^\circ$ .]

*Anal.*—Calcd. for C<sub>31</sub>H<sub>52</sub>O<sub>2</sub>: C, 81.52; H, 11.48. Found: C, 81.25; H, 11.50.

$\gamma$ -Sitosterol Benzoate.—The benzoate was prepared in the usual manner with benzoyl chloride and pyridine. On crystallization from acetone, it gave  $\gamma$ -sitosterol benzoate, m. p. 150–151°,  $[\alpha]_D - 20^\circ$  (CHCl<sub>3</sub>). [Reported (6), m. p. 152°,  $[\alpha]_D - 19.6^\circ$ .]

*Anal.*—Calcd. for C<sub>38</sub>H<sub>54</sub>O<sub>2</sub>: C, 83.34; H, 10.49. Found: C, 82.91; H, 10.45.

### Extraction with Benzene

The defatted plant was next extracted in a Soxhlet apparatus with benzene for four hours. The removal of benzene gave a crystalline residue (9.5 Gm.), which showed a negative test for alkaloids. The residue on crystallization from acetone gave colorless crystalline solids (5.5 Gm.), m. p. 250–256°. The acetone mother liquor on evaporation gave a partially crystalline residue (A) (3.4 Gm.).

Methyl Ursolate.—The crystalline solids (5.5 Gm.), m. p. 250–256°, were esterified with an ethereal solution of diazomethane. The methyl ester was chromatographed over alumina (200 Gm., deactivated with 10 cc. of aqueous 10% acetic acid). Elution with a mixture of petroleum ether and benzene (3:7) afforded a crystalline solid (2.2 Gm.), m. p. 158–165°, which on recrystallization from methanol yielded methyl ursolate, m. p. 168–170°,  $[\alpha]_D + 69.3^\circ$  (CHCl<sub>3</sub>). The mixed melting point with an authentic specimen<sup>1</sup> of methyl ursolate did not show any depression.

Methyl Ursolate Acetate.—Methyl ursolate was acetylated with acetic anhydride and pyridine in the usual manner. The acetate on crystallization from acetone afforded methyl ursolate acetate, m. p. 242–244°,  $[\alpha]_D + 56^\circ$  (CHCl<sub>3</sub>). The mixed melting point with an authentic specimen of methyl ursolate acetate did not show any depression.

*Anal.*—Calcd. for C<sub>33</sub>H<sub>54</sub>O<sub>4</sub>: C, 77.29; H, 10.22. Found: C, 76.83; H, 10.11.

\* Received April 4, 1960, from the Research Division, East India Pharmaceutical Works Ltd., Calcutta-34, India.

The authors wish to express thanks to Mr. Priyalal Moulik for the optical rotation study and to Mr. N. Guha for encouragement.

<sup>1</sup> Kindly supplied by Dr. (Mrs.) A. Chatterjee, Reader in Chemistry, Calcutta University, Calcutta.

**Oleanolic Acid Acetate.**—The residue A (3.4 Gm) from the acetone mother liquor described above was acetylated with acetic anhydride and pyridine. The crude acetate on repeated crystallization from acetone afforded oleanolic acid acetate (0.5 Gm), m p 264–266°,  $[\alpha]_D^{25} + 75^\circ$  ( $\text{CHCl}_3$ ). The mixed melting point with an authentic specimen of oleanolic acid acetate did not show any depression.

*Anal.*—Calcd for  $\text{C}_{32}\text{H}_{50}\text{O}_4$ : C, 77.06, H, 10.11. Found: C, 76.71; H, 10.08.

**Methyl Oleanolate Acetate.**—The acetate was esterified with an ethereal solution of diazomethane and the ester on crystallization from acetone yielded methyl oleanolate acetate, m p 216–218°,  $[\alpha]_D^{25} + 68.6^\circ$  ( $\text{CHCl}_3$ ). The mixed melting point with an authentic specimen did not show any depression.

### Extraction with 95% Ethanol

The plant material after the extraction with benzene, was extracted in a Soxhlet apparatus with 95% ethanol for ten hours. The residue after the removal of ethanol showed a negative test for alkaloids and did not yield any crystalline material.

### REFERENCES

- (1) Chopra, R. N., Nayar, S. L., and Chopra, I. C., "Glossary of Indian Medicinal Plants," Council of Scientific and Industrial Research, New Delhi, India, 1956, p. 180.
- (2) Ratnagiriswaran, A. N., and Venkatachalam, K., *J. Indian Chem. Soc.*, **19**, 389 (1942).
- (3) Chauhan, R. N. S., and Tewari, J. D., *ibid.*, **29**, 386 (1952), **31**, 741 (1954).
- (4) Govindachari, T. R., Nagarajan, K. Pai, B. R., and Rajappa, S., *J. Sci. Ind. Research India*, **17B**, 73 (1958).
- (5) Bhakuni, D. S., *ibid.*, **18B**, 445 (1959).
- (6) "Encyclopedia of Organic Chemistry," Vol. 14, Elsevier Publishing Co. Inc., New York, N. Y., 1940, p. 91.

## Book Notices

*Synthesis and Organisation in the Bacterial Cell* By E. F. GALE. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1959. vii + 110 pp. 12.5 × 18.5 cm.

This is a compilation of material which formed the basis of three Ciba lectures in microbial biochemistry on Structure and organization in the bacterial cell, Amino acid incorporation, and Nucleic acid and protein synthesis.

*The Lynn Index. A Bibliography of Phytochemistry Monograph IV* Organized and edited by JOHN W. SCHERMERHORN and MAYNARD W. QUIMBY. Massachusetts College of Pharmacy, Longwood Ave., Boston 15, Mass., 1960. 70 pp. 15 × 23 cm.

This monograph covers the *Glumiflorae* order, including the Cyperaceae and Gramineae families. The plan for the series was described in the review of monograph I in *THIS JOURNAL*, **47**, 232 (1958).

*Précis D'Anatomie et de Physiologie Humaines* By Y. RAOUL. Masson et Cie, Éditeurs, 120 boulevard Saint-Germain, Paris 6°, France, 1960. viii + 379 pp. 16 × 21 cm. Price NF. 48,000.

This book (in French) presents a concise course in physiology for pharmacy students. It is one of a series of textbooks for the pharmaceutical curriculum.

*Heterocyclic Chemistry*. By ALAN R. KATRITZKY and JEANNE M. LAGOWSKI. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. 274 pp. 13.5 × 20 cm. Price \$4.75.

Designed as a low-cost book for students, the authors have attempted to present a condensed version of basic heterocyclic chemistry required by graduate students and researchers. The authors have been frugal with words but liberal with almost 2,500 formulas.

*Précis de Botanique Vol 2 Systématique des angiospermes* By P. CRÉTÉ. Masson et Cie, Éditeurs, 120 boulevard Saint-Germain, Paris 6°, France, 1960. viii + 429 pp. 16 × 21 cm. Price NF. 34,000.

This second volume on pharmaceutical botanicals (in French) considers the angiosperms (true flowering plants).

*Clinical Chemical Pathology* By C. H. GRAY. Edward Arnold, Ltd., London, England, 1959. Distributed in the U. S. by the Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md. vii + 160 pp. 13 × 18.5 cm. Price \$3.75.

This is the second edition of a pocket-size, handy reference on renal function, acid-base balance, fluid balance (edema) (salt and water deficiencies), liver function tests, chemical pathology of diabetes, Ca and P, disturbances of fat metabolism, chemical pathology of alimentary tract, and biochemical tests in endocrine disease. Other subjects discussed very briefly include special tests and routine clinical tests.

*Antibiotics Annual 1959–1960* Proceedings of the 7th Annual Symposium on Antibiotics. Antibiotica, Inc., 30 East 60th St., New York 22, N. Y., 1960. xvii + 1034 pp. 17 × 25.5 cm. Price \$15.

This volume comprises the proceedings of the seventh annual symposium on antibiotics held November 4–6, 1959, in Washington, D. C. It includes all the reports that were presented. It is, without doubt, the most comprehensive authoritative compilation of research results on new and established antibiotics for the period between the symposiums of 1958 and 1959. It is a must for reference libraries in the health sciences.



*The Clinical Application of Antibiotics. Vol. 4. Erythromycin and Other Antibiotics.* By M. E. FLOREY. Oxford University Press, 417 Fifth Ave., New York 16, N. Y., 1960. viii + 303 pp. 15.5 × 24.5 cm. Price \$19.50.

This volume includes chapters on: Erythromycin; Antibiotics related to erythromycin by reason of bacterial cross resistance; Antibiotics which control the staphylococcus and other Gram-positive bacteria; Antibiotics inhibitory to various bacteria, including Gram-negative organisms; Antibiotics of limited clinical application owing to some toxic effect; Antibiotics active against protozoa, fungi, or neoplastic cells; and a final lengthy chapter on The choice of an antibiotic. Discussions of individual antibiotics follow the same scheme as in previous volumes: general considerations, including properties of clinical significance; antibacterial effects; evidence of toxicity; administration; and results of clinical trials. The book is an authoritative and scholarly reference with appended bibliography and index

*British National Formulary 1960.* Standard Edition. The British Medical Association and the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, 17, Bloomsbury Square, London, W. C. 1, England. Obtainable from Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia 3, Pa., 1960. 272 pp. 10.5 × 16.5 cm. Price 7s 6d. Interleaved, 11s. 6d.

This is the fifth edition of the pocket-size prescribers' formulary. Additions are listed by B. N. F. title and the proprietary name of an equivalent or similar preparation is given.

*Gehes Codex. 9th ed.* Edited by FELIX DIEPENBROCK. Wissenschaftliche Verlagsgesellschaft M. B. H., Stuttgart, and Schwarzeck-Verlag G. M. B. H., Munchen, Germany. Distributed in the U. S. by Texstar Co., 200 West 34th St., New York 1, N. Y., 1960. xi + 1392 pp. 15 × 23 cm. Price \$38.25.

This ninth edition of "Gehes Codex" continues the exceptional coverage of new drug products on the world market, with particular attention to European products, that users of earlier editions have come to expect. An added feature in the latest edition is the indication of trade-marked names. No reference library in pharmacy can be considered complete without the presence of the latest "Gehes Codex."

*Virus Virulence and Pathogenicity.* By Ciba Foundation Study Group No. 4. Little, Brown and Co., 34 Beacon St., Boston 6, Mass., 1960. viii + 114 pp. 12 × 18.5 cm.

This small book includes reports on: The definition and measurement of virus virulence, Host-cell factors and virus virulence, The effect on virulence of changes in parasite and host, Broad aspects of human virulence in influenza viruses, The severity of influenza as a reciprocal of host susceptibility, and The virulence for man of some respiratory viruses passed in tissue cultures. A general discussion and an index are included.

*Basic Facts of Body Water and Ions.* By STEWART M. BROOKS. Springer Publishing Co., Inc., 44 East 23rd St., New York 10, N. Y., 1960. 1f9 pp. 14 × 21 cm. Paperbound. Price \$2.75.

The body's fluid and electrolyte balance in health and in disease states is concisely presented under the two main parts: The facts explained and The facts applied. A glossary and index are appended.

*Handbook of Filtration.* 1st ed. By the technical staff of the Eaton-Dikeman Co. The Eaton-Dikeman Co., Mt. Holly Springs, Pa., 1960. viii + 124 pp. 15.5 × 23.5 cm. Price \$2.50.

This book by the technical staff of The Eaton-Dikeman Co. covers historical development of filtration, paper test methods, retention, permeability, and applications of filter paper. A glossary and index are appended.

*Textbook of Pharmaceutical Chemistry.* 7th ed. By J. E. DRIVER. Oxford University Press, 417 Fifth Ave., New York 16, N. Y., 1960. ix + 728 pp. 15.5 × 24.5 cm. Price \$14.50.

While still maintaining its original design as a student's textbook, the new seventh edition of Bentley and Driver's (Driver sole author since 2nd ed.) is a useful reference for practicing pharmacists and chemists. Part I gives physical and chemical methods of assay for pharmaceutical substances, with detailed applications given in Parts II and III. Part II covers the inorganic materials and Part III is devoted to organic compounds of pharmaceutical importance.

*Methoden der Organischen Chemie (Houben-Weyl).* 4th vol. of the new series. Band V/4, Halogenverbindungen. Edited by EUGEN MÜLLER. George Thieme Verlag, Herdweg 63, Stuttgart-N, West Germany, 1960. Agents in U. S. and Canada, Intercontinental Book Corp., 381 Fourth Ave., New York 16, N. Y. xlvii + 894 pp. Price DM 180, \$42.85.

This volume of a long recognized standard reference series takes up the halogen compounds. The preparation of bromine and iodine compounds and the reactivity and transformation of chlorine, bromine, and iodine compounds are covered. This comprehensive treatise abounds with references to the literature throughout the text, and author and subject indexes are included.

*Chemical Analysis. Vol. 12, Systematic Analysis of Surface-Active Agents.* By MILTON J. ROSEN and HENRY A. GOLDSMITH. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xvii + 422 pp. 15 × 23 cm. Price \$13.50.

This volume treats of a growing and complex group of materials. Surface-active agents are classified and then discussed under the sections: Detection, isolation, and estimation in compositions; Qualitative analysis; Quantitative analysis; Separation of mixtures of surface-active agents. A table of representative commercially-available surfactants is appended and a subject index is included.

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49

SEPTEMBER 1960

NUMBER 9

## Investigation and Development of Protective Ointments III\*

### Adsorption Characteristics of Sarin from Solutions

By K. F. FINGER†, A. P. LEMBERGER, D. E. WURSTER, and T. HIGUCHI

The adsorption of sarin from *n*-heptane solutions by silica gels, alumina, bentonite, and certain carbonaceous adsorbents was studied. The results obtained have been interpreted according to the Langmuir adsorption isotherm. Of the various adsorbents tested, those of a siliceous nature were found to be superior, exhibiting very strong adsorptive tendencies toward the fluorophosphate ester. A linear relationship was found to exist between the limiting adsorptive capacity and the specific surface area of the adsorbents. The average area occupied by a sarin molecule, furthermore, was found to be approximately  $80 \text{ \AA}^2$ , irrespective of the adsorbent, a value in reasonable agreement with that suggested by a molecular model. Both of these relationships indicate that sarin is adsorbed primarily as a unimolecular layer.

THESE INVESTIGATIONS were undertaken to provide basic information pertaining to the nature, intensity, and capacity of certain commercial materials to adsorb sarin.<sup>1</sup> Such information was desirable as a part of the study on protective ointments. It was felt that the information thus obtained would be useful in determining the relationship between the protective capacity of various filler materials and their adsorptive properties. The data, furthermore, were expected to prove useful in developing other protective measures against this and related agents.

This deals with the experimentally determined adsorption behavior of sarin on commercial adsorbents such as silica gels, alumina, bentonite, and certain charcoals from an organic solvent. A method of utilizing this data to predict adsorption behavior from other systems is also discussed.

\* Received August 21, 1959, from the University of Wisconsin School of Pharmacy, Madison.  
Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

This research project was supported by the Directorate of Medical Research, U. S. Army Chemical Warfare Laboratories, Army Chemical Center, Md., under CmlC Contract No. DA18-108-Cml 2576.

† Present address: Charles F. Pfizer Co., Groton, Conn.

<sup>1</sup>Isopropoxymethylphenyl fluoride

### EXPERIMENTAL

The amount of sarin removed from a solution by a solid adsorbent was determined by a relatively simple laboratory procedure which was contingent upon a state of equilibrium being established within the system. A weighed quantity of the adsorbent, approximately 0.1 Gm., was placed in a 25-mm. glass-stoppered weighing bottle and exactly 5 ml. of a known concentration of sarin in normal heptane was pipetted into the bottle. The suspension was then shaken at constant temperature for one hour<sup>2</sup> and then centrifuged. The supernatant liquid was analyzed for its sarin content by the Schoenemann method (2). The weight of sarin adsorbed by 1 Gm. of the adsorbent was calculated from the difference between the initial and equilibrium concentrations of sarin.

The adsorption isotherms were obtained by repeating the above procedure utilizing different initial concentrations of sarin in the solution. All determinations were done in duplicate at 25°. The following adsorbents were studied: silica gels Nos. 21, 63, 72, and 79, SiO<sub>2</sub> (Davison Chemical Co.); Santocel 54, Silica Aerogel (Monsanto Chemical Co.); Sylloid 244, SiO<sub>2</sub> (Davison Chemical

<sup>2</sup> Under the conditions of these experiments, it was previously established that equilibrium was attained within the one-hour period.

Co.); activated alumina (Aluminum Ore Co.); bentonite (Chattanooga Pharmacal Co.); Darco KB and S51, activated charcoals (Darco Division, Atlas Powder Co.); and wood charcoal (J. T. Baker Chemical Co.).

These adsorbents were selected on the basis of their applicability to ointment formulation and also because they were representative of a large number of commercial adsorbents now in use.

## RESULTS AND DISCUSSION

Langmuir's adsorption equation may be stated as

$$x/m = k_1 k_2 c / (1 + k_1 c) \quad (\text{Eq. 1})$$

where  $x/m$  is the weight of adsorbate adsorbed per unit mass of adsorbent,  $c$  is the equilibrium concentration of adsorbate, and  $k_1$  and  $k_2$  are constants. Brunauer has stated (1) that the constants of the Langmuir equation are neither arbitrary nor empirical but, rather, bear physical significance. The adsorption coefficient,  $k_1$ , is related to the force which binds the adsorbate molecules to the adsorbent surface and  $k_2$  is the maximum amount of solute which will be adsorbed per unit mass of adsorbent.

A somewhat more useful form of the Langmuir equation is obtained by inverting Eq. 1 and multiplying through by  $c$ , thus

$$\frac{c}{x/m} = \frac{1}{k_1 k_2} + \frac{c}{k_2} \quad (\text{Eq. 2})$$

A plot of  $c/(x/m)$  versus  $c$  is linear with a slope of  $1/k_2$  and an intercept of  $1/k_1 k_2$ . The adsorption isotherms for several siliceous adsorbents, alumina, bentonite, and three types of carbonaceous adsorbents were plotted according to Eq. 2 and a sample plot is given in Fig. 1.

It was evident from these plots that with all the adsorbents except Darco S51 and wood charcoal, adsorption of sarin obeyed the linear requirements for  $c/(x/m)$  vs.  $c$  plots. A plot of  $x/m$  vs.  $c$  for Darco S51 (Fig. 2) yielded a sigmoid curve characteristic of the Type V isotherm described by Brunauer (3). Although a further study of this system would be of theoretical significance, the extremely low capacity shown by this adsorbent at low sarin concentrations precludes its consideration as a filler material. A similar plot of  $x/m$  vs.  $c$  was made

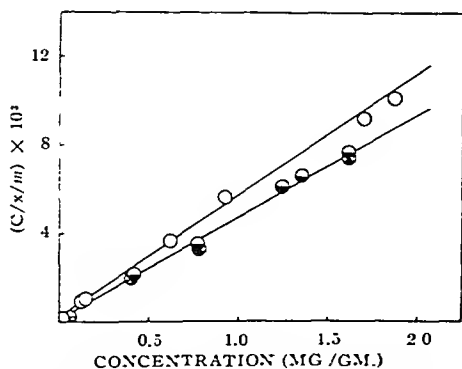


Fig. 1.—Adsorption of sarin from *n*-heptane. O, Silica gel No. 21; ●, silica gel No. 63.

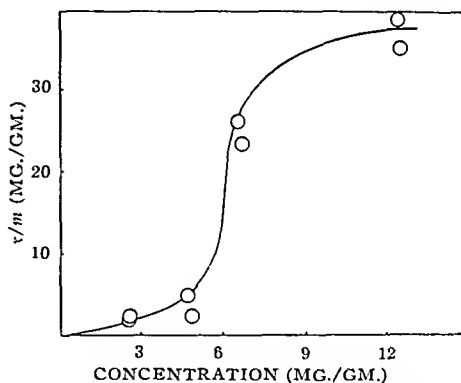


Fig. 2.—The adsorption of sarin from *n*-heptane by Darco S-51.

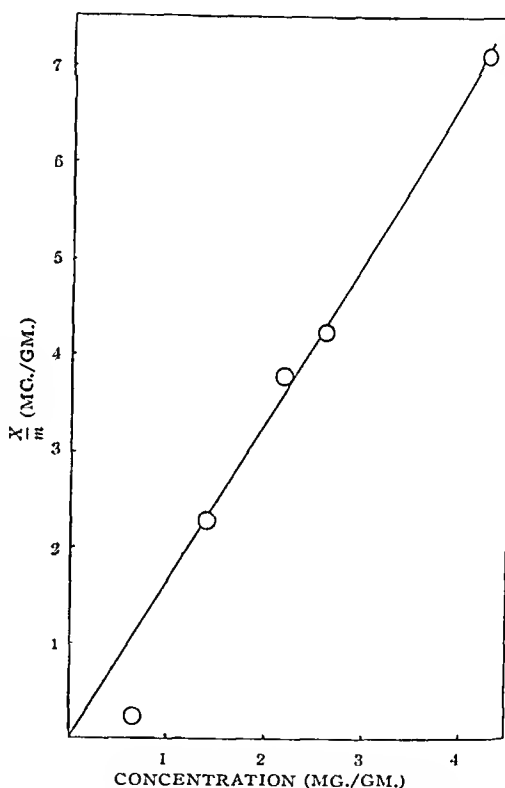


Fig. 3.—Adsorption of sarin from *n*-heptane by wood charcoal.

for wood charcoal (Fig. 3) since the adsorption of sarin was so limited over the concentration range employed that it could not be characterized.

Brunauer has stated that a linear relationship between  $c/(x/m)$  and  $c$  alone is not sufficient evidence that the system follows Langmuir's type adsorption. It is also necessary that the values obtained for the adsorption coefficient and the limiting adsorptive capacity be within reasonable limits (1). Thus, the limiting adsorptive capacity,  $k_2$ , should be proportional to the surface area of the adsorbent if the hypothesis of unimolecular adsorp-

tion is valid and, furthermore, should yield a reasonable value of the area occupied by each adsorbed molecule under close order packing. The adsorption coefficient,  $k_1$ , is dependent upon the nature of the surface and, consequently, should be nearly constant for a homologous series of adsorbents.

The values for the limiting adsorptive capacity,  $k_2$ , for the various adsorbents obtained from the Langmuir plots are listed in Table I. Comparison of these data indicates that the siliceous adsorbents are superior to the other agents tested as adsorbents for sarin.

TABLE I.—PHYSICAL CONSTANTS FOR THE ADSORPTION OF SARIN FROM *n*-HEPTANE

Absorbent	Surface Area, M <sup>2</sup> /Gm.	$k_1 \times 10^{12}$ <sup>a</sup> Gm./mcg.	$k_2 \times 10^{-16}$ <sup>b</sup> mg./Gm.
Santocel 54	175	...	0.38
Sylloid 244	292	6.8	1.20
Silica gel No. 72	300	4.8	0.95
Silica gel No. 79	400	4.9	1.06
Silica gel No. 21	600	6.5	1.84
Silica gel No. 63	800	8.6	2.13
Alumina F-1	200	...	0.58
Bentonite	470	0.18	1.23
Darco KB	600-1,200	0.07	1.49

<sup>a</sup> Adsorption coefficient. <sup>b</sup> Limiting adsorptive capacity

The determination of the  $k_1$  values presented a somewhat greater problem because of the very strong affinity of the phosphate esters for the majority of the adsorbents. Indeed, only approximate evaluation could be made. The usual method of calculating the adsorption coefficients is to determine them from the intercept of the straight line plot of the Langmuir equation. This procedure could not be used effectively in this study because of the close proximity of the interception point with the origin. Instead, an alternate method was employed.

It is evident from the linear Langmuir equation that the adsorption coefficient is described by the following relationship

$$k_1 = \frac{1}{c} \frac{(x/m/k_2)}{1 - (x/m/k_2)} \quad (\text{Eq. 3})$$

where the quantity  $(x/m/k_2)$  is the fraction of the adsorbent's surface occupied by adsorbate molecules and, therefore,  $(1 - x/m/k_2)$  is the fraction left unoccupied. Designating the fraction occupied as  $F_c$  and substituting into Eq. 3, we find

$$k_1 = \frac{1}{c} \frac{F_c}{1 - F_c} \quad (\text{Eq. 3a})$$

It is obvious from the above equation that  $k_1$  is numerically equal to  $1/c$  when the ratio  $F_c/1 - F_c$  is unity.

The procedure followed for the evaluation of the  $k_1$  values, therefore, was to plot  $F_c/1 - F_c$  against the corresponding equilibrium concentrations of the adsorbate as shown in Figs. 4 and 5 and to determine graphically, the concentration when  $F_c/1 - F_c$  was unity. It should be emphasized here that the method required the data to be selected in the range of equilibrium concentrations such that a substantial portion of the surface of the adsorbent

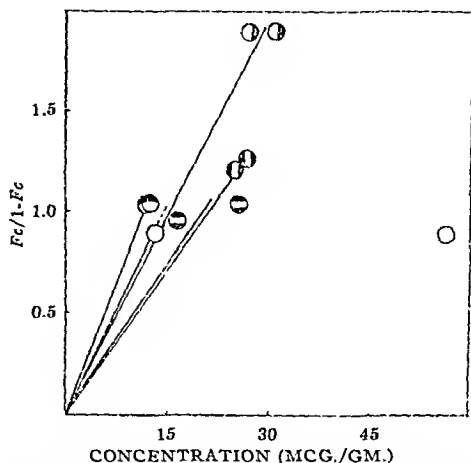


Fig. 4.—Plot to determine the adsorption coefficients of the siliceous adsorbents. ●, Silica gel No. 72; ◐, silica gel no. 79, ◑, silica gel no. 21; ◒, silica gel no. 63; and ○, Sylloid 244.

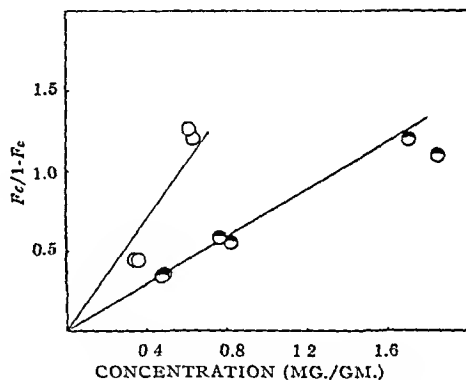


Fig. 5.—Plot to determine the adsorption coefficients. ○, Bentonite; ◐, Darco KB.

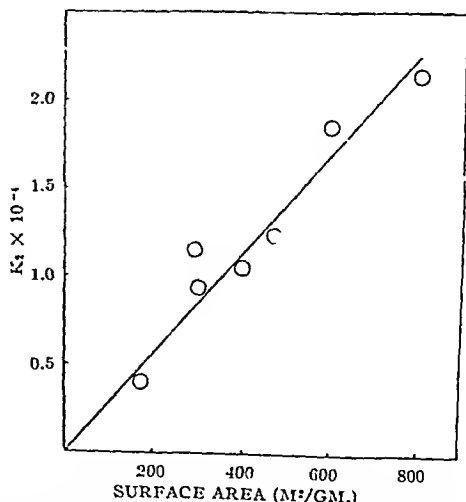


Fig. 6.—Relationship between the limiting adsorptive capacity and the specific surface area of the various adsorbents.

was still uncovered. For adsorbents such as the silica gels, which approach saturation even in very low concentration ranges, considerable error was to be expected. It is evident from the data used in Figs 4 and 5 that only approximate values of the adsorption coefficient can be obtained for the silica gels. These are listed in Table I. The values for bentonite and Darco KB are relatively more dependable.

The question whether these data support the original assumption of formation of a unimolecular layer on the adsorbent surface can be tested by determining the relationship between the available specific surface area of these materials and their limiting adsorption capacity toward sarin.

If the adsorption is unimolecular, the total number of sarin molecules that can be adsorbed will be linearly dependent upon the area of the surface that is available for adsorption. Figure 6 shows the relationship that exists in the systems. The specific surface areas used were those provided by the source of each material. From the slope of this line the average area occupied by an adsorbed molecule can be calculated. The average area calculated for a sarin molecule from the slope of the line was found to be approximately  $80 \text{ \AA}^2$ . To check this value, a scale model of the sarin molecule was constructed and its molecular area roughly determined. The two values were in reasonable agreement.

The adsorption coefficients listed in Table I show a dependence on the chemical nature of the surface. For instance, within a homologous series such as the silica gels, the coefficient assumes an approximately constant value. The surface of the silica gels, alumina, Santocel 54, and Syloid 244 is highly polar in nature while that of the charcoals is relatively nonpolar. Inasmuch as the adsorption coefficient is an index of the primary binding force, the values obtained for the more polar adsorbents should be larger than those obtained for the relatively nonpolar adsorbents. That this relationship holds can be seen by comparing the values of  $k_1$  obtained for the silica gels with those obtained for the charcoals.

While the classification of adsorbents with respect to the polarity of their surface must remain relative, it nevertheless yields some basis for the selection of an adsorbent for a specific adsorbate.

Knowledge of the adsorption characteristics of the penetrant, such as determined in the present study, can be useful information in the formulation of protective ointments. The limiting adsorptive capacities,  $k_2$ 's, of the various adsorbents would necessarily be independent of the solvent employed if solvent adsorbent interactions are negligible. Thus, proper selection of an ointment base would provide a medium for maximum adsorption of the noxious material.

In contrast, the adsorption coefficient,  $k_1$ , is highly dependent upon the solvent from which adsorption occurs. However, in the absence of any strong interaction between solvent and adsorbent, it is possible to obtain a relatively simple relationship between  $k_1$  values for the adsorption of an agent from any two solvent systems by a given adsorbent if the effective partition coefficient of the agent between the two liquids is known. By effective partition coefficient is meant here, the ratio of concentration of the agent present in each solvent when brought into equilibrium with a fixed vapor concentration of the agent. To illustrate, if the partition coefficient of the agent is such that a solvent is favored by a factor of three over the reference solvent, the  $k_1$  value of the agent on any of the adsorbents will be one third that in the reference solvent. Since partition data are readily available, calculations of this type can be carried out for many systems.

It is possible, then, to select a vehicle for the adsorbent in which the adsorption coefficient for the penetrating agent is such that significant retardation of permeation at toxic concentration levels of agent occurs.

## REFERENCES

- (1) Brunauer S. The Adsorption of Gases and Vapors vol. 1 p. 71.
- (2) Koblin A. and Epstein J. *Armed Forces Chem J* 11, 24 (1957).
- (3) Brunauer S. Deming J. S. Deming W. F. and Teller E. *J Am Chem Soc* 62, 1723 (1940).

# Investigation and Development of Protective Ointments IV\*

## The Influence of Active Fillers on the Permeability of Semisolids

By K. F. FINGER†, A. P. LEMBERGER, T. HIGUCHI, L. W. BUSSE, and D. E. WURSTER

The permeability of protective barriers containing adsorbent type fillers has been studied. It has been shown that the time for the nonstationary state permeation of these barriers can be significantly increased by the incorporation of small quantities of an active filler into the ointment base. The theoretical considerations of permeation through filled ointments have been presented with particular emphasis placed on the theory pertaining to barriers containing a filler with a high affinity for the penetrant. An equation relating the lag time to the concentration and maximum adsorptive capacity of this type filler and to the initial concentration of the chemical agent has been derived and verified by experimental procedures.

THE THEORETICAL and experimental investigations of the possible values of active fillers in improving the degree of protection afforded by protective creams and ointments are presented in this report. The term active filler, as used here, includes all fillers which, when incorporated into an ointment or cream base, substantially reduce the chemical or physiological activity of toxic agents by preventing or diminishing the transport of the agent in an active form through the protective barrier.

As pointed out earlier by Higuchi (1), the protective value of creams and ointments during the early stages of penetration does not always correlate with their effectiveness during the later phases. This is especially true with active fillers, the effectiveness of these additives being limited, for all practical purposes, to the initial, nonstationary phase of permeation. For this reason, the present treatment is limited primarily to this initial or nonstationary phase.

### THEORY

The manner in which active fillers manifest their protective action is highly dependent on the way the filler itself reacts with the penetrating agent. It is convenient then for the present purposes to assume two limiting cases for this interaction:

**Case I.**—The thermodynamic activity of the chemical agent in equilibrium with the filler material is considered to be directly proportional to its relative concentration. Thus, if a linear partition

isotherm exists within the system, this condition is satisfied, and

$$a \propto x/m$$

where  $a$  is the activity of the agent and  $x/m$  is the amount of the agent reacted with the filler. This situation would arise, for example, if the filler material was a liquid with a relatively strong affinity for the penetrant. A fair approximation of this case also exists for the adsorbent type filler which possesses a relatively low  $k_1$  value as derived from the Langmuir relationship (2).

**Case II.**—The thermodynamic activity of the penetrant in equilibrium with the filler is considered to be negligibly small until a critical amount of the agent has been taken up. Beyond this point, the activity increases directly with the amount of agent added. This type of behavior exists, in the case of a strongly adsorptive filler which is characterized by a relatively high  $k_1$  value. The filler possessing a high  $k_1$  value will reach a point of saturation at very low equilibrium concentrations of the penetrant.

The mathematical relationships governing the nonstationary state permeation of filled systems considered under case I have been treated in part by Higuchi (1). When a linear partition isotherm exists within a diffusional system, Fick's second law must be modified to account for the amount of penetrant entering the filler phase. The modified equation may be written

$$dc/dt = (D/V_c + KV_f)(d^2c/dx^2) \quad (\text{Eq. 1})$$

where  $V_c$  and  $V_f$  are the volume fractions of the continuous and filler phases, respectively, and  $K$  is the distribution coefficient of the penetrant between the two phases. The movement of the concentration front across the barrier under a driving force of a changing concentration gradient is then described by the following equation

$$C = C_0 \left[ 1 - \frac{2}{\sqrt{\pi}} \int_0^{x/2\sqrt{dt/V_c + KV_f}} e^{-y^2} dy \right] \quad (\text{Eq. 2})$$

where  $C_0$  is the initial concentration of the penetrant in the barrier.

\* Received August 21, 1959, from the University of Wisconsin, School of Pharmacy, Madison.

Presented to the Scientific Section, A. Ph. A. Cincinnati meeting, August 1959.

This research project was supported by the Directorate of Medical Research U. S. Army Chemical Warfare Laboratories, Army Chemical Center, Md., under Cm1C Contract No. DA18-108-Cm1 2576.

† Present address: Charles F. Pfizer Co., Groton, Conn.

It is evident from the above equation that, for a system obeying the conditions given under case I, the lag time will be considerably greater than that obtained for a homogeneous system. Higuchi has stated that this ratio will be equal to  $(V_c + KV_f)$ .

In a homogeneous system, that is, one that does not contain a filler material, the lag time, or time required for the concentration front to move through the barrier, is related to the thickness of the barrier and the diffusion constant by the relationship first derived by Daynes (3) and developed by Barrer (4)

$$L = \frac{x^2}{6D} \quad (\text{Eq. 3})$$

where  $L$  is the lag time,  $x$  is the thickness, and  $D$  is the diffusion constant. Barrer and others have used this relationship to evaluate diffusion constants from permeability data. Thus for a filled system of case I type

$$L = (V_c + KV_f) \frac{x^2}{6D}$$

A similar relationship may be derived for systems containing a filler material which obey the conditions proposed under case II, above. If a linear partition isotherm does not exist within the system, neither Fick's second law nor the modification of it given for case I system is valid; therefore, special boundary conditions must be assumed.

As the penetrant enters the barrier containing a filler with high affinity for the agent, the penetrant is effectively removed from solution by the filler. As a result of the relatively high affinity of the filler for the penetrant, the filler must be saturated before the concentration front is free to progress through the barrier. In the case of the adsorbent type fillers, the above condition implies the maximum adsorptive capacity of the filler must be attained.

Thus, for the experimental system described later in this report, consisting of a barrier membrane whose one surface is brought into equilibrium with the vapor of the penetrating chemical, the rate of movement of the agent through the barrier can be related to other variables in the system in the following manner.

For such a system containing a case II type filler, the concentration gradient of the penetrating chemical in the external phase would approximate that shown in Fig. 1, where the concentration of the penetrating chemical agent within the external phase has been plotted against the distance. For the idealized case where essentially all of the agent entering into the system is found in the filler phase, the gradient, it can readily be shown, will be strictly linear.

It is evident that the total amount of material which has entered such a barrier is the sum of the material found in the external phase and that taken up by the filler. Since the average concentration of the agent up to the point of maximum penetration is  $1/2 C_0$ , where  $C_0$  is the concentration of penetrant in the surface layer exposed to the vapor of the penetrating chemical, the amount in the external phase is equal to  $1/2 C_0 x H$ , where  $x$  is the depth of penetration and  $H$  is the cross sectional area of the barrier.

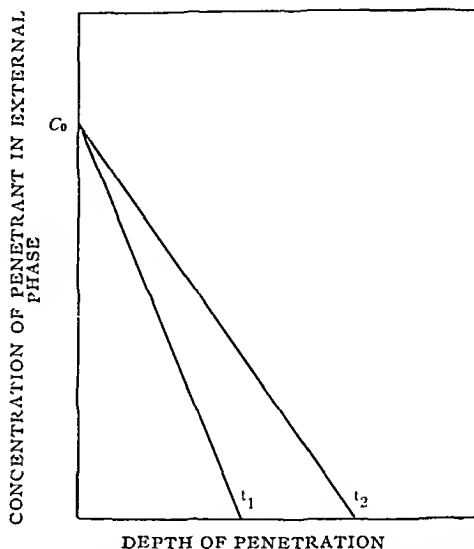


Fig. 1.—Plot of concentration of penetrating chemical in external phase for idealized case II at times  $t_1$  and  $t_2$ .  $C_0$  is the concentration of penetrant in surface layer exposed to the vapor of penetrating chemical.

The amount of material taken up by the filler up to this point, on the basis of previous reasoning, is equal to  $AxH$ , where  $A$  is the amount of penetrant adsorbed by the filler per unit volume of the barrier material. The total amount of penetrant necessary to permit a small additional penetration,  $dm$ , would then be

$$dm = 1/2 C_0 H dx + A H dx \quad (\text{Eq. 4})$$

But since the amount of material crossing a plane of unit area per unit time,  $Q$ ,

$$Q = (dm/dt) = -DH(dc/dx)$$

the equation may then be written

$$\frac{1/2 C_0 H dx + A H dx}{H dt} = -D(dc/dx) \quad (\text{Eq. 5})$$

If the concentration gradient is assumed to be linear over the distance penetrated by the chemical, it is then valid to express the concentration gradient as

$$dc/dx = C_0/x$$

This then leads to the equation

$$\frac{1/2 C_0 H dx + A H dx}{H dt} = -D(C_0/x) \quad (\text{Eq. 6})$$

which may be reduced and rearranged to give

$$\frac{(1/2 C_0 + A) x dx}{C_0} = -D dt \quad (\text{Eq. 7})$$

Or, expressed as an integral equation, it becomes

$$\int_0^x \frac{(1/2 C_0 + A) x dx}{C_0} = -D \int_0^t dt \quad (\text{Eq. 8})$$

The resulting integrated equation then is

$$L = \frac{x^2}{4D} + \frac{Ax^2}{2C_0D} \quad (\text{Eq. 9})$$

where  $L$  represents the time required for the concentration front to move through the barrier, i. e., the lag time.

The amount of penetrant adsorbed by an adsorbent type filler is a function of its maximum adsorptive capacity and thus the quantity  $A$  may be replaced by  $C_f k_2$ , where  $C_f$  is the concentration of the filler and  $k_2$  is the maximum adsorptive capacity of the filler as determined from adsorption experiments (2). The amount of penetrating chemical entering the barrier is governed by the distribution coefficient of the agent between the solvent and the barrier material; then  $C_0$  is equal to  $KC$ , where  $K$  is the distribution coefficient and  $C$  is the initial concentration of the agent in the solvent. Thus, the final equation becomes

$$L = \frac{x^2}{4D} + \frac{C_f k_2 x^2}{2KCD} \quad (\text{Eq. 10})$$

The usefulness of Eq. 10 lies in the fact that the contribution of an adsorptive type filler can be predicted from adsorption data without recourse to the more difficultly obtained permeability data. In the case of the filler being a chemically active detoxicant, a similar equation would result.

It is interesting to note that Eq. 10 predicts a lag time equal to  $x^2/4D$  for the extreme case where none of the penetrant adsorbed, a value which compares with the theoretical lag time for this case,  $x^2/6D$ . Thus, it would seem that Eq. 10 can be expected to give reasonably useful values for lag time even for those cases which deviate appreciably from case II.

## EXPERIMENTAL STUDIES

The influence of the active fillers on the non-stationary permeability of protective barriers was determined by measuring the amount of sarin permeating the barriers as a function of time. The data was then plotted and the lag time for the system determined by extrapolating the straight line portion of the permeability curve to the time axis. In each phase of this work, white petrolatum was used as a control and was also used as the ointment base into which the fillers were incorporated.

**Apparatus.**—The apparatus used in this study was the barrier cell apparatus previously described (5). The bronze washers used were 0.043 cm. thick and possessed an internal diameter of 2.22 cm.

**Preparation of the Ointments.**—A weighed quantity of the melted ointment base, white petrolatum, was placed in a 50-ml. beaker and a weighed quantity of the filler sufficient to produce the desired strength ointment (w/w) was added. The mixture was then stirred to disperse the filler throughout the ointment base. The molten mass was placed in a Bell jar and the system evacuated for at least five minutes to remove any dissolved or adsorbed gases. A second stirring was then initiated and continued until the ointment had congealed. Immediately before the barriers were prepared, the ointment was again evacuated to remove any gases entering the system as a result of the second stirring.

**Preparation of the Barriers.**—The barriers were prepared and mounted in the apparatus in the same manner as previously described (5).

**Experimental Procedure.**—The penetrant utilized in this study was fluorophosphate ester, sarin, dissolved in Amoil-S.<sup>1</sup> After the barrier cell apparatus assembly was completed, exactly 1.5 ml. of the penetrant solution was placed in one of the glass chambers and exactly 1.5 ml. of the pure solvent was placed in the other chamber, i. e., the receiving chamber. The assembly was then placed in a constant temperature bath maintained at 25° and the greased stopcocks were fitted into the glass chambers. Gentle shaking was maintained throughout the experiment by means of a shaking apparatus attached to the bath.

At definite time intervals, 0.1 ml. of the solution in the receiving chamber was removed with a pipet. The sample was placed in a tared, 25-mm. weighing bottle and the weight of the sample determined. The amount of sarin contained in each sample was determined by the Schoenemann method (5) utilizing a Klett-Summerson colorimeter.

The amount of sarin contained in the sample was converted to mcg./Gm. and this quantity was plotted against the time<sup>2</sup> in hours. The lag time for the system was obtained by extrapolating the straight line portion of the permeability curve to the time axis, the point of intersection being the lag time.

## EXPERIMENTAL RESULTS

It has been shown (4) that the lag time is a measure of the rate at which the penetrant molecules diffuse through the barrier material until the concentration front reaches the boundary of the barrier and a pseudo-equilibrium state has been attained. The lag time is therefore a characteristic of the barrier system and may be used as a measure of the protective quality of the system. As a general rule, the longer this lag time, the greater the protective quality of the barrier.

In the permeability curves shown in Figs. 2, 3, 5, and 6, the concentration of sarin permeating the barrier, expressed as mcg./Gm. of solution, is plotted against the time, in hours. The lag time for the particular system may then be obtained by extrapolating the straight line portion of the permeability curve back to the time axis.

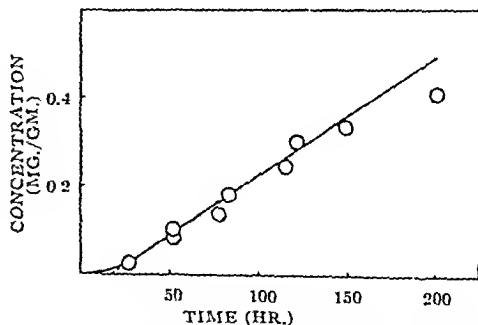


Fig. 2.—Lag time for the permeation of white petrolatum barriers by sarin.  $C = 45.0$  mg./Gm.

<sup>1</sup> n-Amyl sebacate, Central Scientific Co.

<sup>2</sup> It was necessary to introduce a correction for the change of volume of solvent due to the withdrawal of samples. This correction was conveniently made by multiplying the time of sampling by the ratio of the initial volume of solvent to the actual volume remaining after the previous sample had been withdrawn.



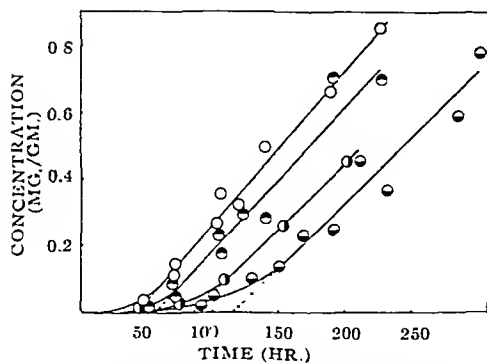


Fig. 3.—Lag time for the permeation of white petrolatum barriers containing 3% w/w of various siliceous adsorbents by sarin.  $C = 45.0$  mg./Gm.; O, silica gel No. 72; ●, silica gel No. 79; ◐, silica gel No. 21; ●, silica gel No. 63.

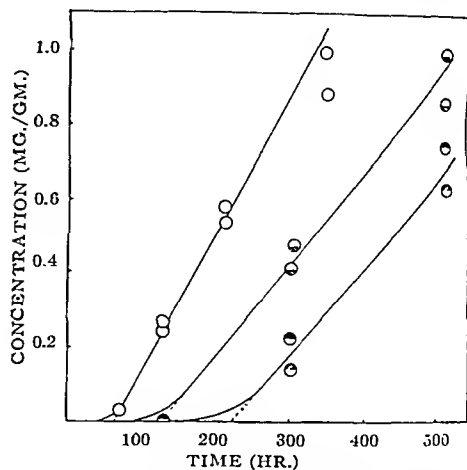


Fig. 5.—Lag time for the permeation of white petrolatum barriers containing various concentrations (w/w) of silica gel No. 72 by sarin.  $C = 10.0$  mg./Gm.; O, 5%; ●, 10%; ◐, 15%.

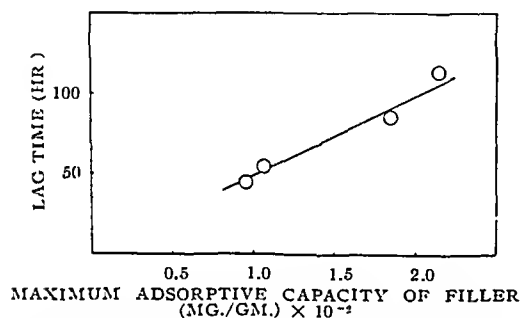


Fig. 4.—Lag time for permeation of a filled ointment barrier as a function of the maximum adsorptive capacity of the filler.

In Fig. 2, the permeability of white petrolatum barriers without a filler is shown. It is evident from the graph that the initial breakthrough of the penetrant occurs at approximately seventeen and one-half hours, or, applying the general rule stated above, the maximum degree of protection afforded by a white petrolatum barrier 0.043-cm. thick would be extended over a period of approximately seventeen and one-half hours.

While the maximum degree of protection afforded by a white petrolatum barrier is of the order of seventeen and one-half hours, the incorporation of as little as 3% (w/w) of an active filler produces a significant increase in the lag time for the system. This is evident in Fig. 3, where the permeability of white petrolatum barriers containing 3% (w/w) of the various siliceous adsorbents is shown.

The lag times for the protective ointments containing the various siliceous fillers are seen to vary with the specific surface area of the filler employed. Thus, lag times ranging from forty-five to one hundred and fifteen hours are observed for the 3% ointments. The maximum lag time, one hundred and fifteen hours, was obtained by using silica gel 63, a siliceous adsorbent possessing a specific surface area of 8002/Gm. This increase in lag time over that of white petrolatum represents an approximate sevenfold increase in the protective quality of the barrier. Proportionately lower

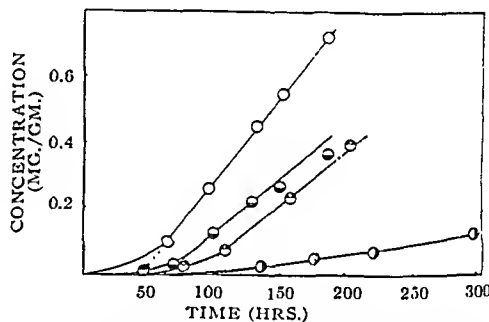


Fig. 6.—Lag time for the permeation of white petrolatum barriers containing 3% w/w silica gel No. 21 utilizing various concentrations of sarin in Amoil-S. O,  $C = 87.3$  mg./Gm.; ●,  $C = 70.3$  mg./Gm.; ◐,  $C = 45.0$  mg./Gm.; ●,  $C = 23.2$  mg./Gm.

protective indexes are observed for other siliceous adsorbents possessing lower specific surface areas.

It has been shown that the maximum adsorptive capacity of a filler is linearly related to its specific surface area (2), that is, the maximum amount of penetrant that will be adsorbed by a filler is dependent upon the amount of surface available for adsorption. Equation 10, derived above, predicts a dependence of the lag time on the maximum adsorptive capacity of the filler. This relationship is shown in Fig. 4. The values for the maximum adsorptive capacity were obtained from adsorption studies previously completed in these laboratories (2) and the lag time values were obtained from Fig. 3.

This relationship between the lag time and the maximum adsorptive capacity is to be expected only in the event that the filler possesses a relatively high affinity for the penetrant. The relative affinity of the adsorbent for the penetrant is readily obtained from adsorption studies. The fact that the proportionality between the lag time and the maximum adsorptive capacity was found to exist

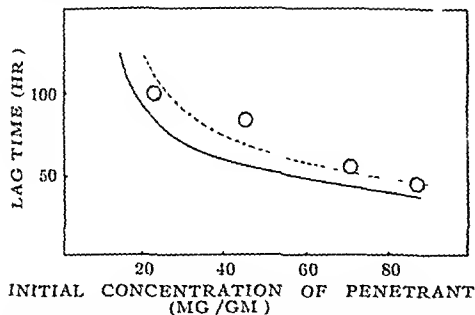


Fig. 7.—Lag time for the permeation of white petrolatum barriers containing 3% (w/w) silica gel No. 21 as a function of the initial concentration of penetrant. Solid line equals theoretical curve

is, in part, verification for the basic assumption of complete saturation of the filler occurring during the nonstationary phase. Further indication that this assumption is valid may be observed in the rather sharp inflection in the permeability curves at the breakthrough point. If a linear partition isotherm existed in the system, a more gradual change in slope would be expected.

The complete saturation of the filler during the nonstationary phase of permeation has an important practical application to protective ointment formulation. If, for example, an ointment base is employed that has a very low affinity for the penetrant, then only a very small amount of the penetrant would be dissolved in the barrier. Inasmuch as a highly active filler must be saturated before the concentration front is free to progress through the barrier and the initial concentration of penetrant is very low, the incorporation of an active filler into such an ointment base would result in an almost indefinite lag time for the system.

While it is advisable to utilize a material possessing a maximum adsorptive capacity as a filler in protective ointments, a similar result may be obtained by increasing the relative concentration of the filler within the ointment. The mechanism of action is identical with that given above. An increase in concentration provides an increased surface area for adsorption and, as a result, the total amount of penetrant that can be adsorbed is

increased. The relationship between lag time and the percentage filler is shown in Fig. 5, where the permeability of white petrolatum barriers containing various concentrations of silica gel No. 72 is shown.

Unfortunately there is a limitation to the amount of filler which can be incorporated into an ointment base since it is possible to incorporate so much filler into an ointment base that cracks or crevices are created which allow the penetrant to travel at an increased rate. This discontinuity in the protective barrier destroys any protective quality imparted to the ointment by the filler.

The initial concentration of penetrant in contact with the barrier is also important in influencing the lag time for the system, as is indicated in Eq. 10, where an inverse dependence of the lag time on the initial concentration is predicted. To substantiate the dependence of the lag time on the initial concentration, experimental determinations of the lag time were made utilizing different concentrations of sarin in Amoil-S as the penetrant. The data for these experiments are plotted in Fig. 6. It is evident from Fig. 6 that an increase in the starting concentration results in a decreased lag time.

It is evident from Eq. 10 that as  $C_0$  becomes very large, the second term loses significance and the lag time approaches a limiting value, namely,  $x^2/4D$ . On the other hand, as  $C_0$  approaches zero, the first term becomes insignificant and the lag time approaches an infinite value.

The agreement between the theoretical and experimental lag times over the range of concentrations employed in this work is shown in Fig. 7. In Fig. 7 the theoretical lag times, as calculated from Eq. 10, appear as a solid line while the experimental values appear as circles, connected by a dotted line. The shape of the experimental curve as compared with the theoretical illustrates a satisfactory agreement between the two.

## REFERENCES

- (1) Higuchi, T., Medical Laboratories Contractor's Report No 32, May 1954
- (2) Finger, K. F., Lemberger, A. P., Wurster, D. E., and Higuchi, T., *THIS JOURNAL*, **49**, 565 (1960)
- (3) Daynes, H., *Proc Roy Soc London*, **97A**, 286 (1920)
- (4) Barrer, R. M., "Diffusion In and Through Solids," Cambridge University Press, England 1951
- (5) Lueck, L. M., *et al*, *THIS JOURNAL*, **46**, 694 (1957)
- (6) Epstein, J. A., *et al*, *J Am Chem Soc*, **78**, 341 (1956).

# The Adsorption of Dodecylbenzenesulfonate and Hexachlorophene on the Skin\*

By GERALD M. COMPEAU

A technique is presented for determining the adsorptive capacity of human skin and the skin of laboratory animals for dodecylbenzenesulfonate and hexachlorophene. The influence of time, pH, and concentration on the adsorption is studied and a mechanism for the adsorption and antibacterial activity of these compounds is postulated.

IT HAS LONG been known that a reaction takes place between anionic surfactants and proteins. The studies of Anson (1), Putnam (2), Putnam and Neurath (3), Valko (4), and others have considered the effect of lauryl sulfate, secondary alkyl sulfate, and dodecylbenzenesulfonate on substrates such as egg albumin, wool, casein, and horse serum albumin.

In recent years, as the incidence of skin irritation (5, 6) has paralleled the increased production and use of synthetic detergents, dermatologists have been concerned with the effect of these compounds on the intact human skin, largely (aside from the gross pathology) from the viewpoint of skin protein denaturation and the accompanying liberation of sulfhydryl groups from the keratin (7, 8, 9). While the older literature stressed the defatting action of the synthetic detergents, their chemical reactivity is now being given more attention.

Although hexachlorophene is almost exclusively used in products formulated to be applied to human skin, its adsorption on the skin surface has received little study. The rationale of its use is based on the fact that continued application of hexachlorophene-containing products reduces the number of viable bacteria which are removable from the skin with Ivory soap by test procedures such as the serial basin technique of Price (10) or modifications thereof (11). Fahlberg, Swan, and Seastone (12) ether-extracted thumbs after the use of hexachlorophene soaps and indirectly estimated the hexachlorophene by a bacteriological assay method.

## EXPERIMENTAL PROCEDURES AND RESULTS

**Materials.**—The hexachlorophene used was the commercial material described in U. S. P. XV. The dodecylbenzenesulfonate was the triethanolamine salt of alkyl (kerosene) benzenesulfonic acid

available as Nacconol 60S.<sup>1</sup> This contains 52% alkyl benzenesulfonate, 8% triethanolamine sulfate and 40% water. It will be referred to as DBSA.

**Determination of Hexachlorophene and DBS on the Skin.**—The method is based on the ultraviolet absorption spectra (Fig. 1) of these compounds in a solvent which is anhydrous methanol (0.015 *M* in phosphoric acid (acid methanol)). The hexachlorophene is determined at 298  $m\mu$  and the DBSA at 262.5  $m\mu$ , correcting for the hexachlorophene found. The determination of the ultraviolet absorption spectra (in this solvent) of many purified dodecylbenzenesulfonates, whether based on *tert*-butyl benzene or tetrapropylbenzene alkylates, shows the presence of fine structure in the beta region of 255–270  $m\mu$  with a maximum at 262.5  $m\mu$ . Reid, Alston, and Young (13) find a  $\lambda_{max}$ , very close to this value for many proprietary products using water as solvent.

For determining the adsorption levels on the skin an extraction technique is used which utilizes a glass-stoppered test tube of known cross-sectional area. To determine the rate of adsorption of hexachlorophene and DBSA as a scrub is carried out, the following procedure is used: obtain a blank value by scrubbing the hands with a brush for several minutes with Ivory soap. With 10 cc. of acid methanol in a test tube (15  $\times$  150 mm., TS 16), extract a dried area of the skin 30 times by inverting the test tube over the skin surface. Scrub for two minutes with the test product and again extract the skin. Continue the scrubs and extractions for the period required and determine the absorbance of the acid methanol extracts at 262.5  $m\mu$  and at 298  $m\mu$  using standardized silica cells of 1.000 cm. ( $\pm 0.5\%$ ) light path.

**Calculations.**—At 298  $m\mu$  the absorption of DBSA is negligible (Fig. 1); thus the absorbance at this wavelength can be taken as a direct measure of hexachlorophene:  $K = A/bc$ , where  $b = 1$  cm and  $c = \text{Gm./L.}$

Since for hexachlorophene  $K_{298} = 14.9$ , and the cell is 1 cm.,  $14.9 = A_{298}/c$ ; and changing from Gm. per liter to mcg. per 10 ml.: mcg. per 10 ml. =  $A_{298} \times 10^4/14.9 \times 10^3$ .

As the 10 ml. contains the hexachlorophene extracted from the cross-sectional area of the test tube (2.12 sq. cm.), hexachlorophene, mcg./sq. cm. =  $A_{298} \times 10^4/14.9 \times 2.12$ .

With the hexachlorophene now being known, the DBSA is determined at 262.5  $m\mu$ , correcting for the absorbance of the hexachlorophene at that wavelength. This correction factor is the ratio  $K_{262.5}/K_{298}$  for hexachlorophene, which is 0.0926.

Then, since  $K_{262.5}$  for DBSA = 0.487,<sup>2</sup> DBSA

<sup>1</sup> Allied Chemical Corp., Buffalo, N. Y.

<sup>2</sup> While this value is for Nacconol 60S, the determination may be made for other alkyl benzenesulfonates if the  $K_{298}$  value is determined for the compound. While all commercial sulfonates contain small amounts of homologs of the dodecylbenzenesulfonate usually considered, the average absorption obtained gives satisfactory values.

\* Received December 31, 1959, from the Research Laboratories, Lehn & Fink Products Corp., Bloomfield, N. J.

$$\text{mcg/sq cm} = A_{260.5} - (A_{298} \times 0.0926) \times 10^4 / 0.487 \times 2.12$$

It has been found that the rate of extraction of the hexachlorophene and DBSA from the skin is such that no significant increase in recovery is obtained beyond 15 extractions and that the 30 extractions used is adequate and requires less than two minutes to carry out. Studies of blank (Ivory) skin extractions show a value for hexachlorophene of about 0.5 mcg/cm<sup>2</sup> and with the lapse of time this value does not change appreciably. A usual blank value for DBSA is 20 mcg/cm<sup>2</sup> and this value will increase slowly with time. The ultraviolet absorption spectra of the blank extracts indicate that this increase in absorption in the DBSA range may be due to the extraction from the skin of tyrosine containing proteins (14).

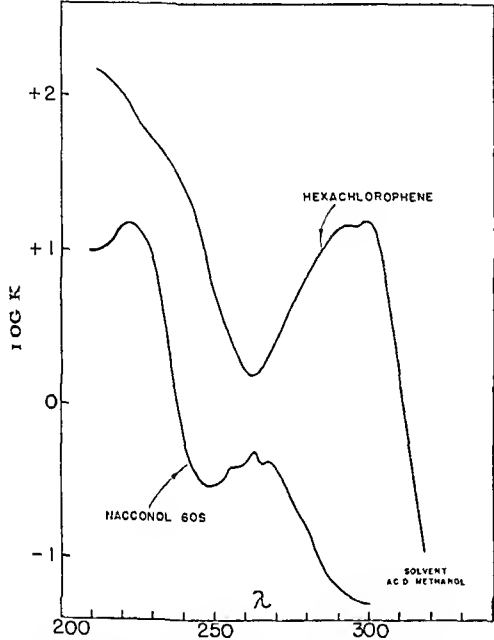


Fig 1—Ultraviolet absorption spectra of hexachlorophene and Nacconol 60S

**Efficiency of the Extraction Technique.**—To estimate the completeness of extraction of the hexachlorophene and DBSA after absorption on a substrate, a scrub series was run using a swatch of undyed Botany worsted wool. This was scrubbed with Ivory soap (blank) and then with scrub No 1681 for five X two minutes. As the scrubs progressed, the wool was extracted as usual. Although an exact comparison cannot be made since the actual area of the wool fibers is not known, the results with the wool are shown in Table I together with the results on the human palm.

TABLE I—ADSORPTIVE CAPACITY OF WOOL		
Substrate	Mcg Adsorbed/Sq Cm Hexachlorophene	Substrate DBSA
Wool (26 mg )	14	135
Human palm	24	570

It is interesting to note that under these conditions the wool has taken up approximately 24% by weight of DBSA calculated as the dry Na salt, and 5% by weight of hexachlorophene. The DBSA value is of the magnitude found by Neville and Jeanson (15) for lauryl sulfate when concentration differences are allowed for. Since the wool showed an appreciable adsorption of both compounds, an effort was made to gauge the completeness of removal of the adsorbed materials. After the twelve minute (cumulative) scrub the wool was sampled, chopped up, and refluxed with acid methanol (i.e., exhaustive extraction) along with an Ivory soap scrubbed piece as a blank. The results are shown in Table II.

TABLE II—RECOVERY OF ADSORBED COMPOUNDS FROM WOOL AFTER EXHAUSTIVE EXTRACTION

Process	Mcg/Sq Cm	
	Hexachlorophene	DBSA
Usual 30 extractions	15	174
Exhaustive extraction	17	270

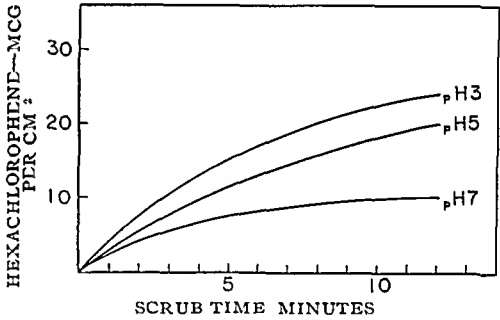


Fig 2—Effect of pH on the adsorption of hexachlorophene on the skin

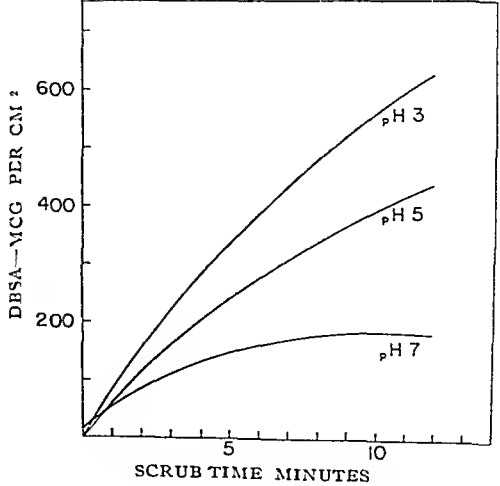


Fig 3—Effect of pH on the adsorption of DBSA on the skin

TABLE III—EFFECT OF pH AND CONCENTRATION ON THE ADSORPTION OF HEXACHLOROPHENE AND DBSA

Surgical Scrub <sup>a</sup>	Hexachlorophene, %	DBSA, %	pH	—Mcg Adsorbed/Sq Cm Skin—	
				Hexachlorophene	DBSA
No 1681	1 0	24	3 0	23	565
No 1692	1 0	24	5 0	19	400
No 1691	1 0	24	7 0	10	185
Phisohex <sup>b</sup>	3 0		5 5	27	
No 1531A	3 0	24	3 0	44	435
No 1291	3 0	48	3 0	48	850
Septisol <sup>c</sup>	0 75		9 4	16	
Gamphen <sup>d</sup>	2 0		9 5	14	

<sup>a</sup> Experimental surgical scrub base = DBSA, lauric diethanolamide 4%, isopropyl lanolin ester 1%, citrate buffer 2% alcohol 12% water

<sup>b</sup> Phiso hex base = sodium octylphenoxxyethyl ether sulfonate, lanolin cholesterol, petrolatum, water.

<sup>c</sup> Septisol base = vegetable oil soap, water

<sup>d</sup> Gamphen base = lanolin 4%, anhydrous soap

The blank (background) correction for DBSA was large on the Ivory-scrubbed wool, indicating a liberation from the wool of an amino acid, probably tyrosine, absorbing in the ultraviolet region of DBSA. The indication is that the results being obtained with the 30-extraction procedure are of the proper order of magnitude.

**Effect of pH and Concentration on Adsorption.**—Using a scrub containing 1% hexachlorophene and 24% DBSA, it can be seen from Figs 2 and 3 that the pH of the scrub product has a pronounced effect on the hexachlorophene and DBSA adsorbed. At low pH, adsorption continues even after prolonged scrub periods, but at pH 7 the rate of adsorption does not increase after eight minutes of scrubbing. For comparative purposes, Table III shows results obtained with several scrubs at a ten-minute cumulative scrub time. These results are shown graphically in Figs 4-6.

At pH 3, and in the presence of 3% hexachlorophene, the DBSA adsorbed on the skin is directly proportional to the concentration in the scrub product, i. e., doubling the concentration increases the DBSA by a factor of 1.96. Hexachlorophene adsorption increases at a somewhat slower rate, tripling the concentration approximately doubles the adsorption.

**Removal of DBSA and Hexachlorophene from the Skin With Soap.**—As shown in Figs 4 and 6, scrubbing with Ivory soap will completely remove DBSA and hexachlorophene from the skin. This removal is complete even after several weeks' exclusive use of these materials.

**The pH of the Skin After Scrubbing.**—The pH of the skin was determined immediately after these scrubs and subsequently to determine to what extent the skin surface pH was maintained. After a six-minute scrub with the buffered product of pH 3, the pH of the skin surface drops to 5.03, from an initial pH of 6.05. This is evidence of the well-known buffer capacity of the skin surface and demonstrates that merely lowering the skin surface pH by 1 unit will highly cationize the skin surface via salt formation at the nitrogen sites, leading to greater adsorption of both hexachlorophene and DBSA. For the effect of soap, Fig 7 shows that the skin surface pH after a six-minute Ivory soap scrub is 7.52 (from 6.05) and that this pH drops off rapidly with time compared to the slower rise after the acid scrubs. Since the products of skin respira-

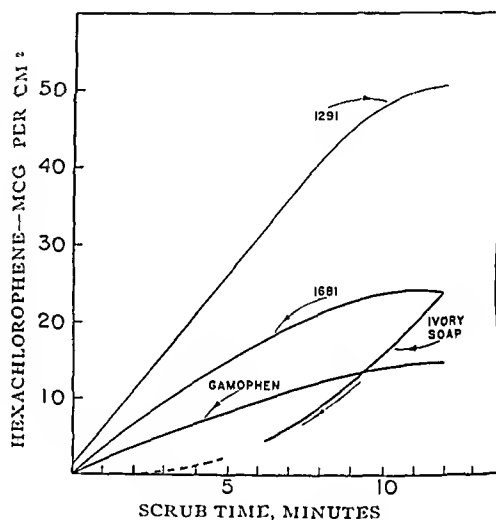


Fig 4—Adsorption of hexachlorophene from various surgical scrubs

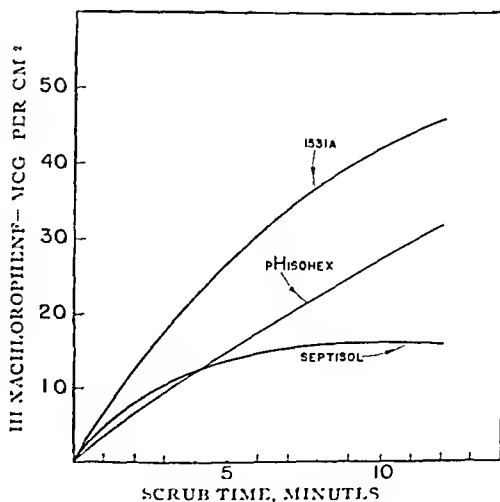


Fig 5—Adsorption of hexachlorophene from various surgical scrubs

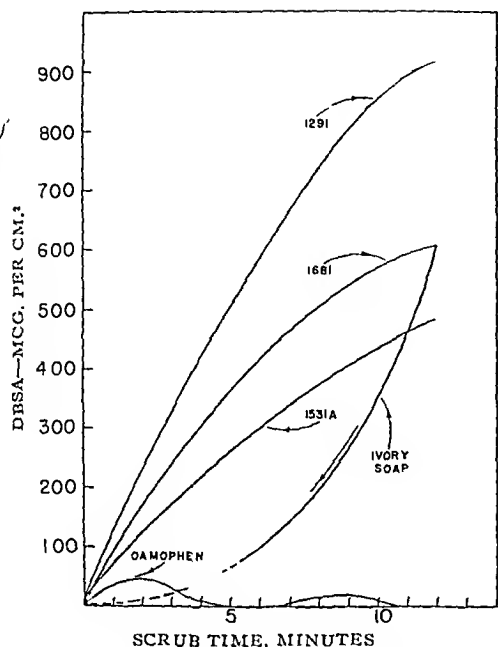


Fig. 6.—Adsorption of DBSA from various surgical scrubs.

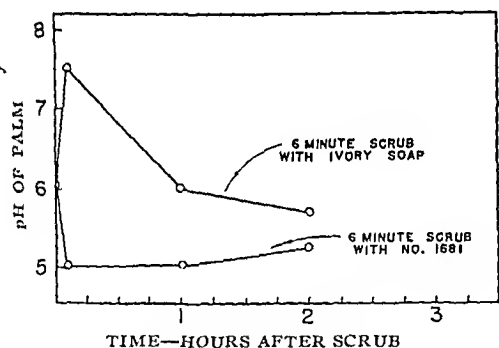


Fig. 7.—The pH of the skin after scrubbing

tion and metabolism are acidic (carbon dioxide, sweat, sebum) these may serve to hasten the more rapid drop in pH (16) after the soap scrub.

**Adsorptive Capacity of Various Skin Sites.**—To evaluate any differences in the adsorptive capacity of various skin areas, and between different species, a series of scrubs was run with the results as shown in Table IV.

This comparison is made at the cumulative ten-minute scrub level, using surgical scrub No. 1681. The results over the full scrub period are shown in Figs. 8 and 9. A great difference in adsorption of hexachlorophene and DBSA on various skin sites is indicated, the most notable difference in man being the much greater adsorption on the more highly keratinized areas of palm and finger tips, compared to the forearm. There is greater adsorption of hexachlorophene on the thumb, and less adsorption on the second finger, when the finger

TABLE IV.—THE ABSORPTIVE CAPACITY OF VARIOUS SKIN SITES

Substrate and Test Area	Mcg. Adsorbed/Sq. Cm. Skin Hexachlorophene	DBSA
Man, palm	23	565
Man, finger tips	24	360
Man, forearm	5	95
Albino rabbit, flank (depilated with thioglycolate)	5	78
Albino rabbit, flank (shaved)	4	0
Albino guinea pig, flank (shaved)	4	20

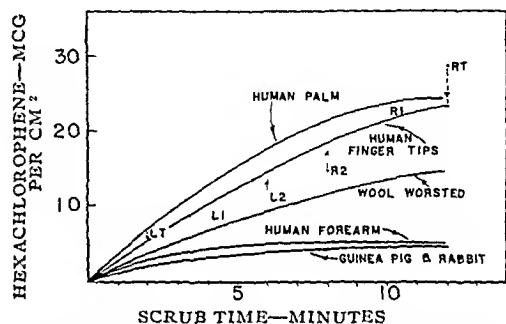


Fig. 8.—Adsorption of hexachlorophene on various substrates. LT = left thumb; L1 = left 1st finger; L2 = left 2nd finger, etc.

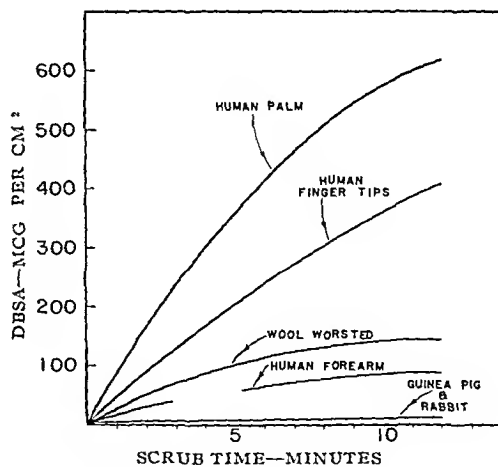


Fig. 9.—Adsorption of DBSA on various substrates.

tips are compared among themselves. The shaved flanks of the rabbit and guinea pig show little adsorption even though some hair is present which cannot be removed without damaging the skin.

**Adsorption Levels With Prolonged Use.**—Hexachlorophene determinations were run on the skin as exclusive scrubbing with a surgical bar soap<sup>3</sup> containing 2% hexachlorophene continued over a period of several days. Thus, experimental data

<sup>3</sup> Gamophen Surgical Soap, Ethicon, Inc., Somerville, N. J.

were gathered which give a basis for comparing the rate of adsorption of hexachlorophene with its rate of disappearance from the skin. The results are shown graphically in Fig. 10. Table V shows comparable results obtained with a low-pH, DBSA-based scrub

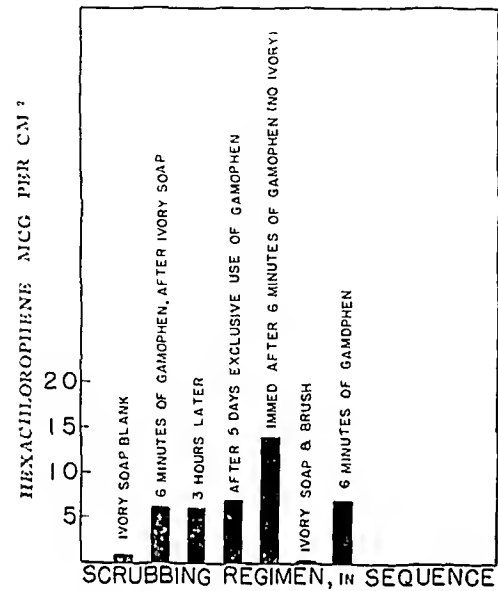


Fig. 10.—The “cumulative” effect of hexachlorophene.

TABLE V.—EXCLUSIVE USE OF HEXACHLOROPHENE SCRUBS

Surgical Scrub	Elapsed Time	Hexachlorophene Absorbed, Mcg./Cm. <sup>2</sup>
Gamophen soap	6 min. (Initial scrub)	6.7
Gamophen soap	5 days	7.1
No. 1681	6 min. (Initial scrub)	16
No. 1681	5 days	15

**Ionic Reaction of Hexachlorophene at Low pH.**—The experiment below was carried out in order to study the extent to which hexachlorophene undergoes ionic reactions in acid solution, and to obtain data useful for postulating a mechanism helpful in considering the substantivity of hexachlorophene to the skin in relation to the skin substantivity of other compounds. Equimolar amounts of the particular compound and a high molecular weight quaternary ammonium chloride compound, Hyamine 2389<sup>1</sup> (C<sub>9</sub> — C<sub>15</sub>-alkyltolylmethyl trimethylammonium chloride) were dispersed in water with the aid of alcohol. The dispersions were diluted out to a concentration of 0.02 *N* and were shaken out once with xylene to remove insoluble material from the reaction mixture. The pH was taken with the glass electrode. The results are shown in Table VI and in Fig. 11.

<sup>1</sup> Rohm & Haas Co., Philadelphia, Pa.

TABLE VI.—REACTION OF ANIONIC COMPOUNDS WITH CATIONIC SURFACTANT

Compound	Molecular Weight	pH Dispersed Without Quaternary	pH Dispersed With Quaternary
Hexachlorophene	407	5.3	2.4
Bithionol	356	6.1	2.2
Oleic acid	282	5.0	3.0
<i>o</i> -Benzyl <i>p</i> -chlorophenol	219	6.6	4.9
<i>o</i> -Phenylphenol	170	6.4	5.8
Cresylic acid	108	6.3	5.8

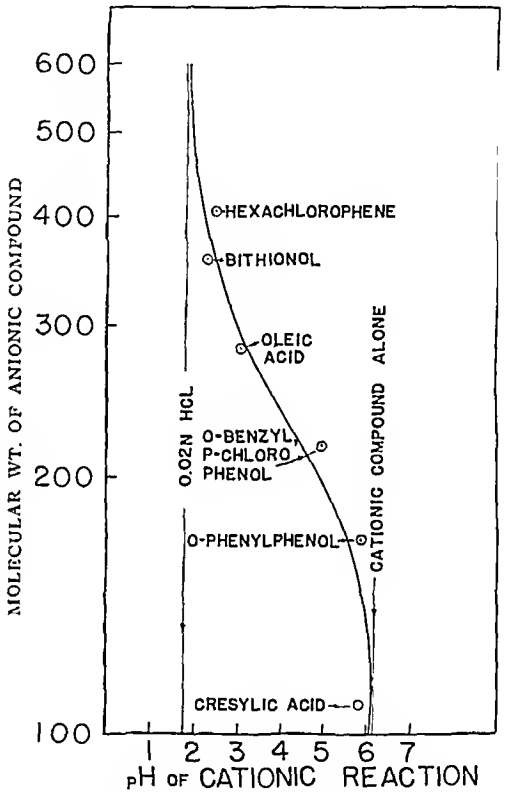


Fig. 11.—Relationship of molecular weight to anion-activity as evidenced by pH drop with cationic compound.

DISCUSSION

**Mechanism of the Adsorption of DBSA.**—From studies carried out with the proteinaceous fibers, silk, wool, and hair (15, 17-19), and with other proteins such as insulin (20), albumin, gelatin, casein, and zein (21), it appears that anionic surfactants can act (as regards adsorption) upon proteins in two ways: (a) at low pH or on the acid side of the isoelectric point of the protein, a straightforward metathetical chemical reaction takes place (chemisorption) between the negatively charged ion of the anionic surfactant molecule and a cationic nitrogen site. Heats of reaction measured are in agreement with this chemisorption reaction; and (b)

on the alkaline side of the protein isoelectric point a physical adsorption takes place with weaker forces holding the protein surfactant together. This would be a weak combination of nonpolar groups of protein and hydrophobic residue of surfactant, similar to the forces of micelle formation (21). The results of this study are in agreement with this theory since an increased degree of substantivity of the DBSA to the skin is found as the pH is lowered. The keratin or other skin protein of the hands follows the general adsorption reactions of proteins and has an isoelectric point, as Rothman (22) points out, probably between pH 4 and pH 5. Both adsorption mechanisms also appear to play a role in the action of the DBSA on bacteria. Flett, *et al.* (23, 24), found that kerylbenzenesulfonate lost most of its bactericidal action in alkaline solution and that the greatest bacterial protein-surfactant combination took place at acid pH. Rapid and complete killing occurred below pH 4. Karabinos (25) envisions the acidified bacterium as being "suffocated" as a result of being "coated" by the anionic surfactant.

**Mechanism of the Adsorption of Hexachlorophene.**—From the structure and ionization constants ( $pK_1 = 5.4$ ;  $pK_2 = 10.9$ ) (26), we might expect the adsorption of hexachlorophene on the skin to be largely of the type discussed in (b) above, but somewhat stronger, perhaps, due to a hydrogen-bonding between phenolic hydroxyl and the  $-NH-$  groups in the protein backbone (27). The results reported here indicate that a common denominator exists between molecular weight, skin substantivity, antibacterial efficacy, and dependence on pH for hexachlorophene and other compounds of its class.

The interrelation of these properties is best understood by considering the anion-active nature of compounds such as benzoic acid, fatty acids, salicylic acid (25), and higher molecular weight phenols on the one hand, and the fact that the skin surface and bacteria (28, 29) are largely proteinaceous and are cation-active at low pH.

For an effective reaction between skin or bacterial protein the anionic reactant must be high enough in molecular weight to be anion-active. This weight is usually such that solubility is low and micelle formation occurs. Kunz and Gump (30) noted that prior to the invention of hexachlorophene, phenols were not suitable as additives (i. e., in small proportion) to soap for the formulation of antibacterial soaps to be used on the skin. It is likely that these phenols were too low in molecular weight for adequate skin substantivity. By the analogy of a quaternary ammonium compound to the cationized skin or bacterial protein, the results given in Fig. 11 show the effect that molecular weight may have (in a series of active compounds) on skin substantivity and antibacterial action. The hexachlorophene, or other compound having a potentially ionizable hydrogen atom, reacts with the quaternary ammonium chloride to yield a high molecular weight, insoluble compound and hydrochloric acid:  $R_4NCl + HO\phi = R_4NO\phi + HCl$ . With a low molecular weight hydrogen ion source, such as cresylic acid, the weight of the anion is too low to form an insoluble compound and only a slight amount of HCl is liberated. As the molecular weight of the anionic compound (hydrogen source) in-

creases, the reaction product with the cationic compound becomes more insoluble and the HCl concentration rises. Since a high molecular weight is necessary for skin substantivity, it can be seen that a scrub product could be effective on inanimate objects where substantivity is less important and yet be unsuitable for use on the skin.

It is well established, in testing phenols for antibacterial activity, that lowering the pH (31) increases the efficacy of the phenol. That is, the killing dilution of phenols becomes much greater at low pH where even such acids as HCl and  $H_2SO_4$  are ineffective by themselves. Since this study has shown that the hexachlorophene adsorbed (Fig. 2) is a function of pH, it would indicate that an ionic mechanism was operating here, i. e., bacteria and the skin surface undergo the reactions of proteins and we cannot consider that we acidify a phenol in the presence of bacteria without recognizing that we acidify the bacterial protein, hence cationize it, and bring about a greater reactivity with the phenol anion. It would be more helpful, then, in understanding the bactericidal process as concerns the relationship between pH and phenols (and other anionic agents) to speak of "cationizing the bacteria" rather than "acidifying the phenol" or other active anion.

In like manner, lowering the pH of the skin increases the cationicity of the skin protein and causes greater reactivity with the hexachlorophene anion. In a dispersed system, even at pH 5 and pH 3, then (in the undissociated form) we can consider hexachlorophene to be an anion-active compound. Also, Pankhurst (27), considering the tanning action of phenols on hides (i. e., collagen) finds there is evidence of ionic interaction in vegetable (phenol) tanning.

**Cumulative Action of Hexachlorophene.**—Workers in the field of degerming (10) the skin frequently speak of a "cumulative action" (32, 33) of hexachlorophene on the skin, by which it is meant, presumably, that the hexachlorophene cumulates on the skin (with exclusive use of a hexachlorophene soap) and builds up or concentrates toward ever-higher levels until a low plateau is reached on the bacterial count-time curve. The actual skin adsorption levels obtained while scrubbing exclusively with a hexachlorophene soap (Fig. 10) may be explained as follows: starting with Ivory soap-clean skin (palms) a six-minute continuous scrub with the surgical bar soap lays down 7 mcg. of hexachlorophene/sq. cm. Three hours later, the hexachlorophene is still about the same level. Five days later, after exclusive use of the soap, the hexachlorophene level is only slightly higher (mid-morning). When this mid-morning skin is scrubbed for six minutes, the adsorption doubles, from 7 to 14 mcg./sq. cm. skin. Vigorous scrubbing with Ivory soap then brings the hexachlorophene down to zero. Again, six minutes with surgical bar soap brings the hexachlorophene back to 7 mcg./sq. cm. skin.

If a cumulation of hexachlorophene were taking place on the skin, then by definition, a significantly higher amount of hexachlorophene per unit area of skin should be present after a given period of time. The results of the present work show that: (a) after five days of exclusive use of the surgical bar



were gathered which give a basis for comparing the rate of adsorption of hexachlorophene with its rate of disappearance from the skin. The results are shown graphically in Fig 10. Table V shows comparable results obtained with a low-pH, DBSA-based scrub

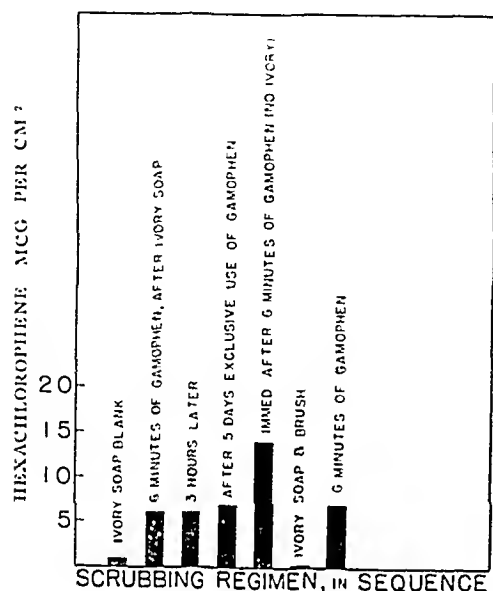


Fig. 10.—The "cumulative" effect of hexachlorophene.

TABLE V—EXCLUSIVE USE OF HEXACHLOROPHENE SCRUBS

Surgical Scrub	Elapsed Time	Hexachlorophene Absorbed, Mcg./Cm. <sup>2</sup>
Gamophen soap	6 min. (Initial scrub)	17
Gamophen soap	5 days	17
No. 1681	6 min. (Initial scrub)	16
No. 1681	5 days	15

**Ionic Reaction of Hexachlorophene at Low pH.**—The experiment below was carried out in order to study the extent to which hexachlorophene undergoes ionic reactions in acid solution, and to obtain data useful for postulating a mechanism helpful in considering the substantivity of hexachlorophene to the skin in relation to the skin substantivity of other compounds.

Equimolar amounts of the particular compound and a high molecular weight quaternary ammonium chloride compound, Hyamine 2389<sup>4</sup> (C<sub>9</sub>—C<sub>15</sub>-alkyltolylmethyl trimethylammonium chloride) were dispersed in water with the aid of alcohol. The dispersions were diluted out to a concentration of 0.02 *N* and were shaken out once with xylene to remove insoluble material from the reaction mixture. The pH was taken with the glass electrode. The results are shown in Table VI and in Fig. 11.

TABLE VI.—REACTION OF ANIONIC COMPOUND WITH CATIONIC SURFACTANT

Compound	Molecular Weight	pH Dispersed Without Quaternary	pH Dispersed With Quaternary
Hexachlorophene	407	5.3	2.4
Bithionol	356	6.1	2.2
Oleic acid	282	5.0	3.0
<i>o</i> -Benzyl <i>p</i> -chlorophenol	219	6.6	4.9
<i>o</i> -Phenylphenol	170	6.4	5.8
Cresylic acid	108	6.3	5.8

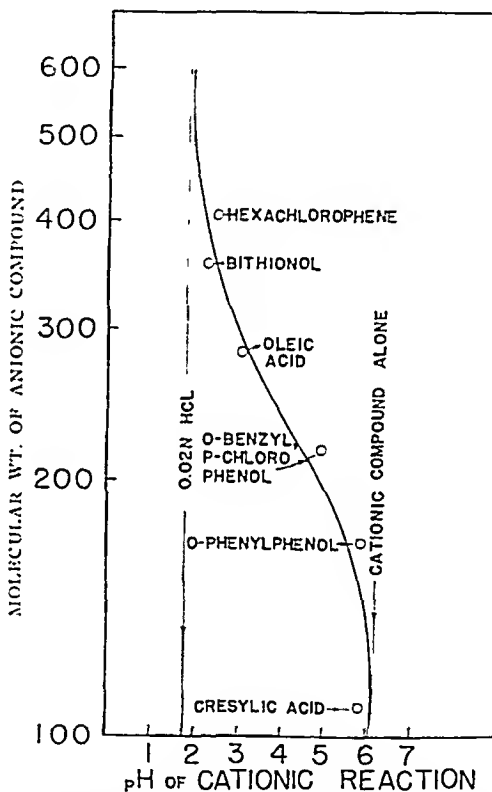


Fig. 11.—Relationship of molecular weight to anion-activity as evidenced by pH drop with cationic compound.

## DISCUSSION

**Mechanism of the Adsorption of DBSA.**—From studies carried out with the proteinaceous fibers, silk, wool, and hair (15, 17-19), and with other proteins such as insulin (20), albumin, gelatin, casein, and zein (21), it appears that anionic surfactants can act (as regards adsorption) upon proteins in two ways: (a) at low pH or on the acid side of the isoelectric point of the protein, a straightforward metathetical chemical reaction takes place (chemisorption) between the negatively charged ion of the anionic surfactant molecule and a cationic nitrogen site. Heats of reaction measured are in agreement with this chemisorption reaction; and (b)

<sup>4</sup> Rohm & Haas Co., Philadelphia, Pa.

on the alkaline side of the protein isoelectric point a physical adsorption takes place with weaker forces holding the protein surfactant together. This would be a weak combination of nonpolar groups of protein and hydrophobic residue of surfactant, similar to the forces of micelle formation (21). The results of this study are in agreement with this theory since an increased degree of substantivity of the DBSA to the skin is found as the pH is lowered. The keratin or other skin protein of the hands follows the general adsorption reactions of proteins and has an isoelectric point, as Rothman (22) points out, probably between pH 4 and pH 5. Both adsorption mechanisms also appear to play a role in the action of the DBSA on bacteria. Flett, *et al.* (23, 24), found that kerylbenzenesulfonate lost most of its bactericidal action in alkaline solution and that the greatest bacterial protein-surfactant combination took place at acid pH. Rapid and complete killing occurred below pH 4. Karabinos (25) envisions the acidified bacterium as being "suffocated" as a result of being "coated" by the anionic surfactant.

**Mechanism of the Adsorption of Hexachlorophene.**—From the structure and ionization constants ( $pK_1 = 5.4$ ;  $pK_2 = 10.9$ ) (26), we might expect the adsorption of hexachlorophene on the skin to be largely of the type discussed in (b) above, but somewhat stronger, perhaps, due to a hydrogen-bonding between phenolic hydroxyl and the  $-NH-$  groups in the protein backbone (27). The results reported here indicate that a common denominator exists between molecular weight, skin substantivity, antibacterial efficacy, and dependence on pH for hexachlorophene and other compounds of its class.

The interrelation of these properties is best understood by considering the anion-active nature of compounds such as benzoic acid, fatty acids, salicylic acid (25), and higher molecular weight phenols on the one hand, and the fact that the skin surface and bacteria (28, 29) are largely proteinaceous and are cation-active at low pH.

For an effective reaction between skin or bacterial protein the anionic reactant must be high enough in molecular weight to be anion-active. This weight is usually such that solubility is low and micelle formation occurs. Kunz and Gump (30) noted that prior to the invention of hexachlorophene, phenols were not suitable as additives (i. e., in small proportion) to soap for the formulation of antibacterial soaps to be used on the skin. It is likely that these phenols were too low in molecular weight for adequate skin substantivity. By the analogy of a quaternary ammonium compound to the cationized skin or bacterial protein, the results given in Fig. 11 show the effect that molecular weight may have (in a series of active compounds) on skin substantivity and antibacterial action. The hexachlorophene, or other compound having a potentially ionizable hydrogen atom, reacts with the quaternary ammonium chloride to yield a high molecular weight, insoluble compound and hydrochloric acid:  $R_4NCl + HO\phi = R_4NO\phi + HCl$ . With a low molecular weight hydrogen ion source, such as cresylic acid, the weight of the anion is too low to form an insoluble compound and only a slight amount of HCl is liberated. As the molecular weight of the anionic compound (hydrogen source) in-

creases, the reaction product with the cationic compound becomes more insoluble and the HCl concentration rises. Since a high molecular weight is necessary for skin substantivity, it can be seen that a scrub product could be effective on inanimate objects where substantivity is less important and yet be unsuitable for use on the skin.

It is well established, in testing phenols for antibacterial activity, that lowering the pH (31) increases the efficacy of the phenol. That is, the killing dilution of phenols becomes much greater at low pH where even such acids as HCl and  $H_2SO_4$  are ineffective by themselves. Since this study has shown that the hexachlorophene adsorbed (Fig. 2) is a function of pH, it would indicate that an ionic mechanism was operating here, i. e., bacteria and the skin surface undergo the reactions of proteins and we cannot consider that we acidify a phenol in the presence of bacteria without recognizing that we acidify the bacterial protein, hence cationize it, and bring about a greater reactivity with the phenol anion. It would be more helpful, then, in understanding the bactericidal process as concerns the relationship between pH and phenols (and other anionic agents) to speak of "cationizing the bacteria" rather than "acidifying the phenol" or other active anion.

In like manner, lowering the pH of the skin increases the cationicity of the skin protein and causes greater reactivity with the hexachlorophene anion. In a dispersed system, even at pH 5 and pH 3, then (in the undissociated form) we can consider hexachlorophene to be an anion-active compound. Also, Pankhurst (27), considering the tanning action of phenols on hides (i. e., collagen) finds there is evidence of ionic interaction in vegetable (phenol) tanning.

**Cumulative Action of Hexachlorophene.**—Workers in the field of degerming (10) the skin frequently speak of a "cumulative action" (32, 33) of hexachlorophene on the skin, by which it is meant, presumably, that the hexachlorophene cumulates on the skin (with exclusive use of a hexachlorophene soap) and builds up or concentrates toward ever-higher levels until a low plateau is reached on the bacterial count-time curve. The actual skin adsorption levels obtained while scrubbing exclusively with a hexachlorophene soap (Fig. 10) may be explained as follows: starting with Ivory soap-clean skin (palms) a six-minute continuous scrub with the surgical bar soap lays down 7 mcg. of hexachlorophene/sq. cm. Three hours later, the hexachlorophene is still about the same level. Five days later, after exclusive use of the soap, the hexachlorophene level is only slightly higher (mid-morning). When this mid-morning skin is scrubbed for six minutes, the adsorption doubles, from 7 to 14 mcg./sq. cm. skin. Vigorous scrubbing with Ivory soap then brings the hexachlorophene down to zero. Again, six minutes with surgical bar soap brings the hexachlorophene back to 7 mcg./sq. cm. skin.

If a cumulation of hexachlorophene were taking place on the skin, then by definition, a significantly higher amount of hexachlorophene per unit area of skin should be present after a given period of time. The results of the present work show that: (a) after five days of exclusive use of the surgical bar

soap there is no increase in concentration of hexachlorophene on the skin; the rate of use of the surgical soap is counterbalanced by the rate of disappearance; (b) a higher level of hexachlorophene is attained when the soap is used on a normal (acid) skin than on an alkaline (Ivory-serubbed) skin. It would appear that the concentration of hexachlorophene on the skin is not so much a function of the number of days elapsed since the first scrubbing was done as it is on the condition of the skin, length of scrubbing time, and the time-lapse since the previous scrub. The largest use of hexachlorophene is in soaps intended to be used over an extended period of time, and practically all of the bacteriological work done which considers the "cumulative" effect of hexachlorophene is on soap-based products. Although low-pH, DBSA-based surgical scrubs lay down on the skin far more hexachlorophene than a soap-based product in a given period of scrubbing as indicated in Table I, it is interesting to note that determinations of hexachlorophene on the palms of four subjects using surgical scrub No. 1681 exclusively for a period of twenty-one days show that there is no cumulation of hexachlorophene at all on the hands over this extended period of time; there is no more hexachlorophene on the skin on the twenty-first day than there was on the first day. Thus, there is no cumulation of hexachlorophene on the skin from either an alkaline soap or an acid syndet.

It follows, then, that the antibacterial effectiveness of hexachlorophene on the skin is not due to a cumulative building-up of this compound on the skin, but rather to its being maintained at a given level on the skin for a long period of time. According to Cade (11) the efficacy of hexachlorophene on the skin lies in its being continuously available in sufficient concentration to lessen the degree of multiplication of the bacteria. It is theoretically possible that the concentration of hexachlorophene on the skin resulting from a single scrubbing with a hexachlorophene soap might be sufficient to obtain a low bacterial count after a lapse of several days if we did not have to be concerned with this concentration being lowered through being rubbed off on clothing, being inactivated (34) or diluted by perspiration or other body exudations, etc. Continuous use of hexachlorophene soap may serve more the purpose of providing a modest concentra-

tion of "fresh" hexachlorophene to a site where the hexachlorophene applied earlier had become inactivated or "spent" than the purpose of "building-up" the hexachlorophene concentration to a high level.

## REFERENCES

- (1) Anson, M. L., *J. Gen. Physiol.*, **23**, 239(1939); **24**, 399(1941).
- (2) Putnam, F. W., *Advances in Protein Chem.*, **4**, 79(1948).
- (3) Putnam, F. W., and Neurath, H., *J. Am. Chem. Soc.*, **66**, 692(1944).
- (4) Valko, E. I., *Ann. N. Y. Acad. Sci.*, **46**, 451(1946).
- (5) Schneider, W., *Soap, Perfumery & Cosmetics*, **26**, 15(1953).
- (6) Stüpel, H., *Seifen-Ole-Fette-Wachse*, **83**, 604(1957).
- (7) Van Scott, E. J., and Lyon, J. B., *J. Invest. Dermatol.*, **21**, 109(1953).
- (8) Van Scott, E. J., and Flesch, P., *Arch. Dermatol. and Syphilol.*, **70**, 141(1954).
- (9) Harrold, M. A., *J. Invest. Dermatol.*, **32**, 581(1959).
- (10) Price, P. B., *J. Infectious Diseases*, **63**, 301(1938).
- (11) Cade, A. R., *Soap Sanit. Chemicals*, **26**, 36(1950).
- (12) Fahlberg, W. J., Swan, J. C., and Seastone, C. V., *J. Bacteriol.*, **56**, 323(1948).
- (13) Reid, V. W., Alston, T., and Young, B. W., *Analyst*, **80**, 682(1955).
- (14) Holiday, E. R., *Biochem. J.*, **30**, 1800(1936).
- (15) Neville, H. A., and Jeanson, C. A., *J. Phys. Chem.*, **37**, 1001(1933).
- (16) Szakall, A., *Arbeitsphysiologie*, **11**, 436(1941).
- (17) Aicken, R. G., *J. Soc. Dyers Colourists*, **60**, 60, 170(1944).
- (18) Flett, L. H., Hoyt, C. F., and Walter, J. E., *J. Am. Oil Chemists' Soc.*, **32**, 166(1955).
- (19) Meender, A. L., and Fries, B. A., *Ind. Eng. Chem.*, **44**, 1644(1952).
- (20) Miller, G. L., and Andersson, K. J., *J. Biol. Chem.*, **144**, 475(1942).
- (21) Lundgren, H. P., *Textile Research J.*, **15**, 335(1945).
- (22) Rothman, S., "Physiology and Biochemistry of the Skin," Univ. of Chicago Press, Chicago, Ill., 1954, pp. 11, 230.
- (23) Flett, L. H., *Oil & Soap*, **22**, 245(1945).
- (24) Flett, L. H., Gusteras, A. F., Haring, R. C., and Shapiro, R. L., *Am. Perfumer & Essent. Oil Rev.*, **48**, 63(1946).
- (25) Karabinos, J. V., and Ferlin, H. J., *J. Am. Oil Chemists' Soc.*, **31**, 228(1951).
- (26) Mahler, W., *J. Am. Chem. Soc.*, **76**, 3920(1954).
- (27) Pankhurst, K. G. A., "Surface Phenomena in Chemistry and Biology," Pergamon Press, New York, N. Y., 1958, p. 115.
- (28) Porter, J. R., "Bacterial Chemistry and Physiology," John Wiley & Sons, New York, N. Y., 1946, p. 359.
- (29) Baumgärtel, T., "The Newer Knowledge of Bacteriology and Immunology," University of Chicago Press, Chicago, Ill., 1928.
- (30) Kunz, E. C., and Gump, W. S., U. S. pat. 2,535,077(1950).
- (31) Goedrich, P., *This Journ.*, **27**, 1233(1938); **30**, 85(1941).
- (32) Havens, I., Benham, R. S., and Clark, D. E., *Am. J. Med. Technol.*, **23**, 76(1957).
- (33) Best, R. R., Coe, J. D., McMurtrey, G. B., and Henn, M. J., *Arch. Surg.*, **61**, 869(1950).
- (34) Cade, A. R., and Gump, W. S., "Antiseptics, Disinfectants, Fungicides, and Sterilization," Lea & Febiger, Philadelphia, Pa., 1957, p. 330.

# Distribution Coefficients and Dissociation Constants of a Series of Barbituric Acid Derivatives\*

By J. P. LEYDA, D. J. LAMB, and L. E. HARRIS

An equation was derived to illustrate the relationship between distribution coefficients and dissociation constants of weak acids. The method used in determining the distribution coefficients of a series of barbituric acid derivatives is described. These data obtained from the distribution studies are used to calculate the thermodynamic dissociation constants of a series of barbituric acid derivatives. The relationship and methods employed are shown to be valid and the results reproducible.

THIS INVESTIGATION was undertaken as the preliminary phase of a study for determining a possible relationship between free energy differences and biological activity. It was the purpose of this investigation to study the relationship between the distribution coefficients and dissociation constants of some barbituric acid derivatives.

Distribution studies have been used to determine dissociation constants of various organic acids and bases (1, 2). Golumbic (1) has derived an equation to illustrate the relationship between distribution coefficients and dissociation constants in a system when the hydrogen ion concentration is insignificant in comparison to the dissociation constant. This equation is not applicable to systems in which the hydrogen ion concentration and dissociation constant are of the same magnitude.

The dissociation constants of barbituric acid derivatives have been determined by Krahf (3), using pH measurements at various ionic strengths, and by Biggs (4), using spectrophotometric measurements at various hydrogen ion concentrations.

## THEORY

During this investigation it was necessary to derive an equation to illustrate the relationship between the distribution coefficient and the dissociation constant of a weak acid. An assumption is made and the terms of the system defined. It is assumed that the weak acid is monomeric in the

aqueous and organic phases and only the undissociated species distributes between the immiscible solvents.

In this equation,  $k$  is the observed ratio of the concentration of weak acid in the aqueous phase to its concentration in the organic phase at a specific pH,  $k_0$  is a similar observed ratio at a pH where only the undissociated acid is present, and  $pK_a$  is the logarithm of the reciprocal of the dissociation constant of the acid.

The following equation can be derived from the relationships between  $k$ ,  $k_0$ , and the Henderson-Hasselbalch equation.

$$pK_a = pH - \log (k - k_0)/k_0$$

From this equation the apparent dissociation constant of a weak acid can be calculated from the distribution coefficient of the acid at a pH where only undissociated molecules are present and at a pH where partial dissociation of the acid is possible. See Table I.

The true thermodynamic dissociation constant which is based on the activity of the acid may be calculated when the acid concentration is converted into terms of activity. The correction factor used in changing to activity is the mean activity coefficient ( $-\log f_{\pm}$ ).

$$pK_a' = pK_a - \log f_{\pm}$$

This correction factor is the same as used by Biggs (4). The mean activity coefficient can be calculated by the Davies equation (5).

## EXPERIMENTAL

**Materials.**—The barbiturates were recrystallized from ethanol-water mixtures with the exception of butethal. Due to the low melting point of this compound a mixture of ether-petroleum ether (30–60° b. p.) was employed. Tris (hydroxymethyl) amino methane, commonly referred to as "tris," was purified by the method described by Fossum (6).

The chloroform used in these studies was purified by washing with a sodium bicarbonate solution, distilled water, and a saturated solution of sodium chloride, respectively. It was partially dried with the addition of anhydrous magnesium sulfate and then distilled. The isooctane used was of reagent grade and the water employed was demineralized and double distilled.

**Spectrophotometric Assay.**—In solutions of a pH of 9 or below barbiturates have extremely low extinction coefficients. With the addition of an alkali to the aqueous barbiturate solutions to give a pH of about 11 the absorption increases due to the enol formation (7).

A known concentration of barbiturate was dissolved in an alkaline buffer with the pH of 10.9. The wavelength of maximum absorption

\* Received August 21, 1959, from the Ohio State University, School of Pharmacy, Columbus 10.

This investigation was supported by a grant from the National Institutes of Health, Public Health Service.

Abstracted from a thesis presented to the Graduate School of the Ohio State University by James P. Leyda in partial fulfillment of the requirements for the degree of Master of Science.

This paper was the winner of the Lunsford Richardson Pharmacy Award (Eastern Division).

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

TABLE 1.—DATA PERTAINING TO A SERIES OF BARBITURIC ACID DERIVATIVES USED IN THE DERIVED EQUATION

Generic Name	R	R'	Max. $\mu$	Molar Extinction, $E \times 10^{-3}$	$k_a$	$k$	pKa Found	pKa Lit
Barbital	Et	Et	239	10.2	9.06	21.62	8.06	7.97 (4)
Probarbital	Et	isopropyl	240	9.8	2.49	5.12	8.17	8.01 (3)
Butethal	Et	<i>n</i> -butyl	239	11.5	0.47	0.99	8.10	7.92 (3), 7.98 (4)
Butabarbital	Et	<i>sec</i> -butyl	240	11.0	0.46	0.96	8.16	....
5-Ethyl-5-(2-methyl)-allylbarbituric acid	Et	2-methyl allyl	240	9.6	1.60	5.29	7.85	....
Pentobarbital	Et	1-methyl butyl	240	10.1	0.17	0.35	8.17	8.11 (3)
Vinbarbital	Et	1-methyl butenyl	239	9.5	0.64	2.58	7.72	....
Amobarbital	Et	isoamyl	239	10.0	0.16	0.40	8.02	7.96 (4), 7.94 (3)
Cyclobarbital	Et	cyclo-1-enyl	239-240	10.6	0.61	2.10	7.79	7.50 (3)
Phenobarbital	Et	phenyl	239	11.0	1.58	9.14	7.54	7.45 (3)
Diallylbarbituric acid	Allyl	allyl	241	9.2	2.30	7.25	7.88	7.77 (3)
Aprobarbital	Allyl	isopropyl	241	9.2	1.32	3.09	8.09	7.99 (3)
Allylbarbituric acid	Allyl	isobutyl	240	9.5	0.41	1.35	7.86	....

and the molar extinction coefficients were determined by measuring the absorbance of known concentrations of barbiturates at 239, 240, and 241  $\mu$ . The measurements were obtained by the use of the Beckman DU spectrophotometer.

These data were used in assaying the aqueous phases during the distribution studies.

**Buffer Solutions.**—The preparation of the tris and acidic buffer solutions of a definite ionic strength followed a modification of the general scheme shown by Bates (8). The alkaline buffer solutions were prepared with the weak base tris, hydrochloric acid, and either potassium or sodium chloride added to give the desired ionic strength. The acidic buffers were prepared using the hydrochloric acid and sodium or potassium chloride to adjust the ionic strength. The ionic strength of the buffer solutions were calculated in the usual manner.

The ultimate standard employed in determining pH was a phosphate buffer described by Bates (9). The pH of the tris buffer solutions were determined at 25° with a Beckman GS pH meter.

**Distribution Studies.**—The solvent system used was an aqueous buffer phase and a mixture of chloroform (60%) and isooctane (40%). To determine the distribution coefficient ( $k_o$ ) of the undissociated acid the acidic buffer was used as the aqueous phase. The tris buffer at a known pH was used as the aqueous phase in determining the distribution coefficient ( $k$ ).

A stock solution of barbiturate (approx.  $9 \times 10^{-4}$  but known) was prepared in the buffer solutions. Equal portions of the organic phase and the stock barbiturate solution were placed in 10-dram Opticlear vials. The vials were sealed and placed in a constant temperature bath at  $25 \pm 0.01^\circ$  to equilibrate. When temperature equilibrium was reached the vials were shaken for one minute. The phases were allowed to separate completely and then an aliquot of the aqueous phase was removed, diluted with pH 10.9 buffer to give a suitable absorbance, and measured spectrophotometrically. The original stock solution was assayed in a similar manner.

The following equation was used to calculate the  $k$  and  $k_o$ .

$$k \text{ or } k_o = \frac{A \text{ aqueous phase after distribution}}{A \text{ stock solution} - A \text{ aqueous phase}}$$

where  $A$  represents the absorbance. Direct substitution of the measured pH,  $k$ , and  $k_o$  into the derived equation gave the apparent pKa' of the barbiturate. Upon correction to activities by the Davies equation the true thermodynamic pKa was obtained. The ionic strength of the aqueous buffer solutions was 0.02.

## DISCUSSION AND RESULTS

The equation which was derived to correlate the relationship between the distribution coefficients and the dissociation constants of weak acids was based on the assumption that the acid was present in the monomeric form in the aqueous and organic phases and only the undissociated species distributes between the immiscible solvents. This assumption was shown to be valid due to the agreement between the literature dissociation constants and the experimentally determined values.

This equation is more applicable than previously derived relationships since the hydrogen ion concentration can be of the same or different magnitude than that of the dissociation constant. The pH of the aqueous phase has no confined limits as long as the acid is monoprotic. In systems which contain a nonmonoprotic acid the pH must be maintained at a value where it can be assumed that the effect of other ionizable groups is insignificant.

## SUMMARY

1. An equation was derived to correlate the relationship between distribution coefficient and dissociation constants of weak acids.

2. The wavelength of maximum absorbance and the molar extinction coefficients of thirteen barbiturates were determined.

3. The dissociation constants of a series of barbituric acid derivatives were calculated. The distribution coefficients of the barbiturates were determined at 25° in a system of aqueous solution, and a mixture of 60 per cent chloroform and 40 per cent isoctane.

4. The assumption on which the relationship between distribution coefficients and dissociation constants of weak acids was based was shown to be true.

## REFERENCES

- (1) Golumbic, C., Orchin, M., and Weller, S., *J. Am. Chem. Soc.*, **71**, 2624 (1949).
- (2) Wall, F. T., *ibid.*, **64**, 472 (1942).
- (3) Krahf, M. E., *J. Phys. Chem.*, **44**, 449 (1940).
- (4) Biggs, A. I., *J. Chem. Soc.*, 1956, 2485.
- (5) Davies, J., *ibid.*, 1938, 2093.
- (6) Fossum, J. H., Markunas, P. C., and Riddick, J. A., *J. Anal. Chem.*, **23**, 491 (1951).
- (7) Stuckey, R. E., *Quart. J. Pharm. and Pharmacol.*, **14**, 217 (1941).
- (8) Bates, R. G., "Electrometric pH Determinations," John Wiley & Sons, Inc., New York, N. Y., 1954, p. 117.
- (9) Bates, R. G., and Acree, S. F., *J. Research Natl. Bur. Standards*, **30**, 129 (1943).

## Correlation of the Distribution Coefficients of Various Barbituric Acids\*

By DONALD J. LAMB and LOYD E. HARRIS

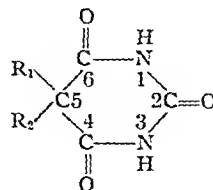
Evidence is provided to indicate that the distribution coefficients of various barbituric acids can be related to the number of carbon atoms and the Taft polar constants of the 5,5-substituents. This suggests that the hydrophilic portion of the molecule is affected not only by the size of the substituents but, also, by their polarity.

series of barbituric acids as a function of inductive or polar effects as well as the number of carbon atoms in the 5,5-substituents of these compounds.

## DISCUSSION

If a series of compounds, such as 5,5-substituted barbituric acids, is chosen which differ from one another only in carbon content of their substituents, it would be expected that the distribution of these compounds would change with the alteration of the lipophilic character of the substituents as well as the influence these groups exert on the rest of the molecule.

This influence exerted by the substituents on the rest of the molecule may be in the nature of resonance, steric, or inductive (polar) effects. In the 5,5-substituted barbituric acids used in this study the 5-position of the barbituric acid ring is saturated and, therefore, would be incapable of transmitting any resonance effects from the substituents to the carbonyl groups in the ring.



Here the carbonyl groups, in positions 4 and 6, are defined as the "reaction sites." Since the 5,5-substituents are not adjacent to these reaction sites, it would seem that steric inhibition of solvation of the hydrophilic portion of the molecule would be

ATTEMPTS have been made to develop relationships between changes in structure and the distribution of compounds between an organic solvent and water. Collander (1) tabulated numerical estimates of the effects of various groups on the distribution coefficients of organic compounds. Alders (2) showed the logarithm of the distribution coefficient to be a linear function of the number of carbon atoms in the alkyl groups of a series of homologous straight-chain organic compounds. However, he reported that deviations from linearity do occur when the alkyl chain contains less than three or four carbon atoms. This, he postulated, is due to differences in inductive effects in lower members of the alkyl side-chain and this difference rapidly decreases as the number of carbon atoms increases.

The purpose of this paper is to develop a relationship of the distribution coefficients of a

\* Received August 21, 1959, from the College of Pharmacy, The Ohio State University, Columbus 10.

This paper was awarded honorable mention in the Lunsford Richardson Pharmacy Award Contest.

Presented to the Scientific Section, A Ph A., Cincinnati meeting, August 1959.

TABLE I—CORRELATION OF THE EFFECT OF STRUCTURE ON THE DISTRIBUTION COEFFICIENTS OF 5,5-SUBSTITUTED BARBITURIC ACIDS BY EQ 1

Compound	R <sub>1</sub>	R <sub>2</sub>	N	$\Sigma\sigma^*$	Log K	
					Observed	Calcd
1 Barbitol	C <sub>2</sub> H <sub>5</sub>	C <sub>2</sub> H <sub>5</sub>	4	-0.225	+0.957	+0.958
2 Probarbitol	C <sub>2</sub> H <sub>5</sub>	<i>i</i> -C <sub>3</sub> H <sub>7</sub>	5	-0.240	+0.396	+0.331
3 Butethal	C <sub>2</sub> H <sub>5</sub>	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	6	-0.215	-0.328	-0.242
4 Butabarbital	C <sub>2</sub> H <sub>5</sub>	C <sub>2</sub> H <sub>5</sub> (CH <sub>2</sub> )CH	6	-0.255	-0.337	-0.296
5 Pentobarbital	C <sub>2</sub> H <sub>5</sub>	C <sub>3</sub> H <sub>7</sub> (CH <sub>2</sub> )CH	7	-0.255	-0.770	-0.842
6 Amobarbital	C <sub>2</sub> H <sub>5</sub>	<i>i</i> -C <sub>3</sub> H <sub>7</sub>	7	-0.215	-0.796	-0.788
7 Phenobarbital	C <sub>2</sub> H <sub>5</sub>	C <sub>6</sub> H <sub>5</sub>	8	+0.04	+0.199	+0.199
8 Aprobarbital	CH <sub>2</sub> =CHCH <sub>2</sub>	<i>i</i> -C <sub>3</sub> H <sub>7</sub>	6	-0.185	+0.121	+0.082
9 Allylbarbituric acid	CH <sub>2</sub> =CHCH <sub>2</sub>	<i>i</i> -C <sub>3</sub> H <sub>7</sub>	7	-0.190	-0.387	-0.491
10 Diallylbarbituric	CH <sub>2</sub> =CHCH <sub>2</sub>	CH <sub>2</sub> =CHCH <sub>2</sub>	6	-0.120	+0.362	+0.431
11 5-Ethyl 5-(2-methyl) allylbarbituric acid	C <sub>2</sub> H <sub>5</sub>	CH <sub>2</sub> =C(CH <sub>3</sub> )CH <sub>2</sub>	6	-0.175	+0.204	+0.135

slight or at least constant for the series studied. Inductive or polar effects then would be the main effects exerted by the substituents.

If these assumptions are considered valid, the following equation may be written:

$$\log K = \gamma + \rho \Sigma \sigma^* + \log K_0 \quad (\text{Eq. 1})$$

where  $K$  is the distribution coefficient of the barbituric acid between aqueous acid solution and 40% iso-octane-chloroform solution. These data and methods for their determination are reported elsewhere (3).  $\log K$  is the distribution coefficient of the unsubstituted barbituric acid,  $N$  is the number of carbon atoms in the 5,5-substituents, and  $\Sigma\sigma^*$  is the sum of the Taft polar constants (4). Gamma and  $\rho^*$  are "reaction constants" indicating the susceptibility of the reaction or distribution coefficient to a change in the number of carbon atoms and polarity, respectively.

Equation 1 is analogous to that reported by Taft (6) to describe the rates of methanolysis of a series of *l*-methyl esters in terms of the polar and steric effects of the acyl component of the esters.

The polar constants for the series of eleven 5,5-substituted barbituric acids listed in Table I were obtained from published data (6). They were chosen considering the 5,5-substituents and the carbon atom in position five of the ring as a unit, i.e., the polar constant for aprobarbital (5-allyl-5-isopropylbarbituric acid) was obtained from the sum of the polar constants for the CH<sub>2</sub>=CHCH<sub>2</sub>-CH<sub>2</sub>- and (CH<sub>3</sub>)<sub>2</sub>CH-CH<sub>2</sub>- groups rather than the allyl and isopropyl groups. This is justified in that the value listed for the (C<sub>2</sub>H<sub>5</sub>)CH- group is -0.225 while doubling the value of the *n*-propyl group leads to -0.230. Since the polar constant for a 3-butenyl group was not available, its value was calculated from the pK<sub>a</sub> of vinylacetic acid (7) and the equation describing the ionization constants of carboxylic acids as a function of the polar constants of their substituents (5). The value obtained was +0.17, the value of the CH<sub>3</sub>CH=CH- group is +0.36, and that for the CH<sub>3</sub>CH=CHCH<sub>2</sub>- group is +0.13, therefore, introduction of a methylene group decreases the constant by 0.23. Combining this with the value of +0.17 for the allyl group leads to a value of -0.06 for the CH<sub>2</sub>=CHCH<sub>2</sub>-CH<sub>2</sub>- group. By similar methods a value of -0.055 was obtained using the pK<sub>a</sub> of allylmalonic acid (7) after first subtracting the constant of the HOOCCH<sub>2</sub>- group.

The number of carbon atoms of the 5,5-substituents were used without addition of the carbon in position five of the ring as a matter of expedience since addition of a constant number to the value of  $N$  would have no overall effect.

The constants of Eq. 1 were determined by standard statistical procedures as conveniently outlined by Taft (6). The values obtained along with their standard deviations are  $\gamma = -0.5462 \pm 0.0006$ ,  $\rho^* = 5.38 \pm 0.09$ , and  $\log K_0 = 4.353 \pm 0.036$ . The multiple correlation coefficient is 0.997 and the probable error of the fit of a single observation is 0.08 log unit. The values of  $\log K$  calculated by this equation are listed in Table I together with the experimentally determined values.

It is evident that the distribution coefficients of the 5,5-substituted barbituric acids are affected by more than the change in carbon number of their substituents (see Fig. 1). When a correction is applied for the polar effects of the substituents, a linear relationship is obtained (Fig. 2). Considering this linear relationship and the magnitude of the

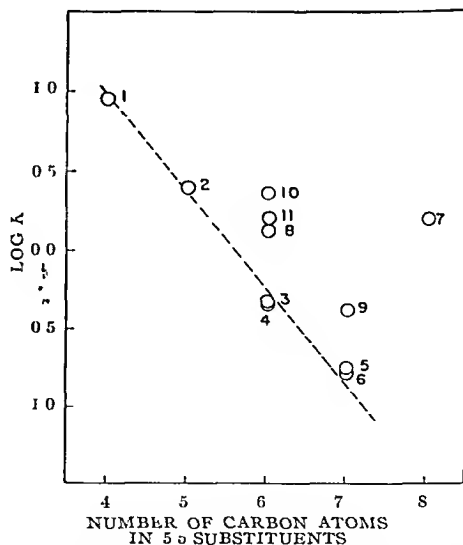


Fig. 1—Failure of single parameter to correlate the distribution coefficients of 5,5-substituted barbituric acids. The numbers adjacent to the circles correspond to the compounds listed in Table I.

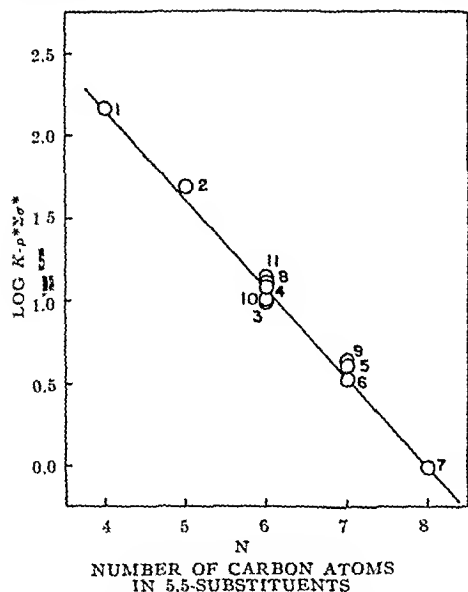


Fig. 2.—Correlation of the distribution coefficients of 5,5-substituted barbituric acids by the equation  $\log K - \rho^* \Sigma \sigma^* = \gamma N - \log K_0$ . The numbers adjacent to the circles correspond to the compounds listed in Table I.

polar reaction constant ( $\rho^* = +5.38$ ), small changes in the inductive effects of the lipophilic portion of the molecule can have a pronounced effect on the reaction site or hydrophilic portion of the molecule. The excellent correlation of these two parameters with the experimental data would indicate that steric effects, as expected, are constant or negligible in this reaction series.

Equation 1 emphasizes the fact that the carbon atom number and the polar effects are independent of each other, i. e., if one is constant or zero, the other defines the changes in  $\log K$ . This is illustrated in Fig. 2 with compounds 3, 4, 8, 10, and 11 all having a value of  $N = 6$  and the polar constant

changing by a factor of more than 2. The fit of the data to the theoretical line is in good agreement as predicted by the equation. On the other hand, when the polar constants are nearly constant as in compounds 1, 2, 3, 4, 5, and 6, a linear relationship is obtained (Fig. 1) from the plot of  $\log K$  versus number of carbon atoms.

Equation 1 can be used with some confidence in predicting the distribution coefficients of barbituric acid derivatives substituted in position five by hydrocarbon groups provided the polar constants are available or a reasonable estimate can be made.

## SUMMARY

An equation has been derived to establish the distribution coefficients of a series of 5,5-substituted barbituric acids as functions of the number of carbon atoms and Taft polar constants of their substituents. The correlation coefficient indicates an excellent fit of the data to the equation with a probable error of a single observation of 0.08 log unit. This indicates that the distribution of the barbiturates is affected not only by the mass of the 5,5-substituents but, also, by the influence of the inductive effects of these groups on the hydrophilic portion of the molecule. It has also been shown that the two parameters which define the distribution coefficients are independent of each other as predicted by Eq. 1.

## REFERENCES

- (1) Collander, R., *Acta Chem Scand*, **3**, 717 (1949).
- (2) Alders, L., *Appl. Sci. Research*, **A4**, 171 (1954).
- (3) Leyda, J. P., Lamb, D. J., and Harris, L. E., *THIS JOURNAL*, **49**, (581) 1960.
- (4) Newman, M. S., "Steric Effects in Organic Chemistry," John Wiley & Sons, Inc., New York, N. Y., 1956, p. 619.
- (5) *Ibid*, p. 607.
- (6) Pavelich, W. A., and Taft, R. W., Jr., *J. Am. Chem. Soc.*, **79**, 4935 (1957).
- (7) International Crit. Tables, Vol. VI, McGraw-Hill Book Co., Inc., New York, N. Y., 1929, p. 259.



# An Evaluation of Certain Hypotensive Agents IV\*

## Diquaternarized Piperidine Derivatives

By J. P. BUCKLEY, M. L. JACQUART, R. K. BICKERTON†, W. J. HUDAK‡, F. M. SCHALIT†, J. J. DEFEO§, and J. A. BIANCULLI

Certain diquaternarized piperidine derivatives and several bis-tertiary amines were evaluated in the anesthetized rat and dog. The most active hypotensive agents were: 3-morpholinopropyl N-methylpiperidinium dimethiodide (JB-643), 2-morpholinoethyl N-methylpiperidinium dimethiodide (JB-654), and beta(3-diethylamino-1-propynyl)-N-methylpiperidine dimethiodide (JB-579). The compounds JB-643 and JB-654, administered orally to unanesthetized dogs, produced marked hypotensive responses persisting for more than eleven hours.

CERTAIN diquaternarized piperidinecarboxylic acid esters have previously been reported by our laboratory to be potent hypotensive agents (1-3). Beta-dimethylaminoethyl N-methylpiperidinium (JB-591) has also been reported to possess both central and peripheral hypotensive properties and to be effective in controlling essential hypertension in human patients (2). This present report deals principally with the evaluation of additional diquaternarized piperidinecarboxylic acid esters, certain of their "reversed" esters, and some  $\beta$ -dialkylaminoalkynyl-N-methylpiperidines (Fig. 1).

### EXPERIMENTAL

**Hypotensive Activity in Rats.**—The experimental compounds were screened and evaluated in anesthetized normotensive rats using the method described in a previous paper (4).

The oral activity of JB-654 was also investigated. The compound was administered via gastric intubation to rats prepared as follows:

(a) Rats were anesthetized with urethan, 1.2 Gm./Kg., intraperitoneally and the carotid blood pressures were recorded on a Sanborn Viso-Cardiette utilizing a Sanborn electromanometer.

(b) Blood pressures of unanesthetized rats were determined via a photoelectric tensometer (Metro Industries) or by direct recording utilizing the method of Still, *et al.* (5). Direct blood pressures were obtained via a small length of polyethylene

tubing (PE-10, Clay Adams) permanently inserted into the abdominal aorta.

**Hypotensive Activity in Dogs.**—Several of the compounds were further evaluated in normotensive dogs to study the possibility of species variation.

(a) Mongrel dogs were anesthetized with pentobarbital sodium, 35 mg./Kg., i. v., and the blood pressures were recorded via a femoral artery. Fresh solutions of the compounds were administered via a femoral vein.

(b) The oral activity of JB-643 and JB-654 was investigated in unanesthetized normotensive dogs. Mongrel dogs were anesthetized with 150 mg./Kg. of hexobarbital sodium, i. v. An incision approximately 1 cm. in length was made through the skin just above and perpendicular to the femoral artery. A segment of the artery, approximately 4 cm. long, was carefully isolated and a sterile nasal oxygen catheter (Bardie 10) inserted into the lumen to an approximate depth so that it entered the abdominal aorta and was affixed by a double ligature. The catheter had previously been cut to the proper length, filled with sterile heparin solution (1,000 U. S. P. units per ml.), and the plastic adapter and cap, or B-D L/609 adapter and 417A male plug, inserted. The catheter was sutured to the skin of the leg, the incision closed, and the entire leg wrapped with sterile gauze and then with an elastic bandage. Procaine penicillin G, 100,000 units, was administered intramuscularly into the opposite leg and the dog was permitted to recover for a minimum period of two days before being utilized. The dogs were immobilized by suspending them in a canvas sling from a suitable height. Direct blood pressures were obtained by connecting a sterile saline system to the nasal catheter and were recorded via a Sanborn electromanometer or a mercury U-tube manometer. The compounds were administered in capsule form to dogs fasted for twenty-four hours.

### RESULTS AND DISCUSSION

**Hypotensive Activity in Rats.**—The hypotensive activity of the compounds, administered intravenously, is summarized in Table I. The most potent compounds investigated in this present series were JB-643 and JB-654 (Fig. 2), two diquaternarized esters of the piperidinecarboxylic acid. This agrees with the results obtained in our laboratory with a similar series of compounds (1), in that the diquaternarized esters of piperidinecarboxylic acid, having the side chain containing two or three methylene units ( $\text{CH}_2$ ) attached to a quaternarized dimethylamino or morpholino group linked in the 2-position of the piperidine moiety, were the most

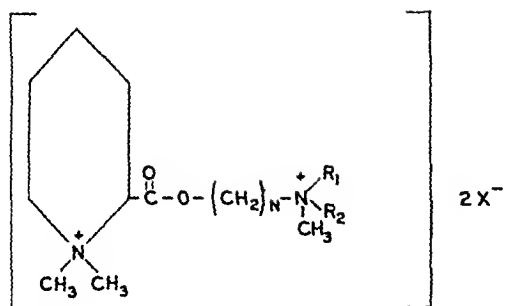
\* Received August 21, 1959, from the University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

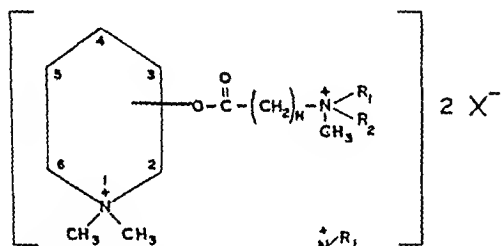
This investigation was supported in part by a research grant from Lakeside Laboratories, Inc., Milwaukee, Wis. The experimental compounds were kindly supplied by Dr. H. L. Daiell, Scientific Director, Lakeside Laboratories, Inc.

† George A. Kelly, Sr., Fellow.  
‡ Present address: Department of Pharmacology, Wm. S. Merrell & Co., Cincinnati, Ohio.

§ Present address: School of Pharmacy, University of Rhode Island, Kingston.



COMPOUND JB-	N	$\begin{array}{c} \text{R}_1 \\   \\ \text{N}^+ \\   \\ \text{R}_2 \\   \\ \text{CH}_3 \end{array}$	$\text{X}^-$
608	3	$\text{N}(\text{CH}_3)_2$	I
643	3	MORPHOLINO	I
654	2	MORPHOLINO	BR



COMPOUND JB-	POSITION	N	$\begin{array}{c} \text{R}_1 \\   \\ \text{N}^+ \\   \\ \text{R}_2 \\   \\ \text{CH}_3 \end{array}$	$\text{X}^-$
592	3	1	$\text{N}(\text{CH}_3)_2$	BR
607	3	2	$\text{N}(\text{CH}_3)_2$	I
647	3	1	PYRROLIDINO	I
642	3	2	MORPHOLINO	I
646	4	1	$\text{N}(\text{CH}_3)_2$	BR

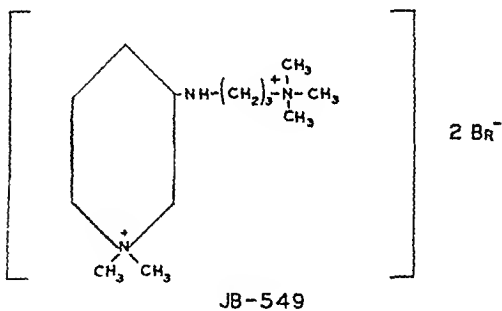
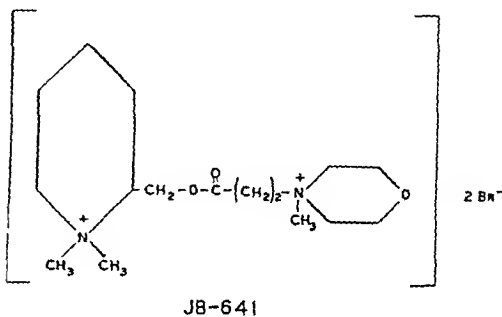
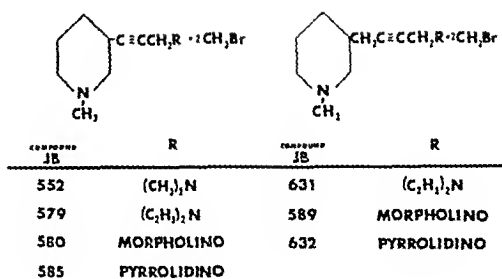


Fig. 1.—Structures of the compounds investigated

active compounds. It appears that the replacement of the terminal dimethylamino group (JB-591) with a morpholino radical (JB-654) not only maintained but also increased the hypotensive activity. The "reversed" esters of N-methylpiperidinecarboxylic acid derivatives, which were investigated, had the side chain linkage at the 3 or 4 position. The most potent compounds were the morpholinoethyl (JB-642) and pyrrolidinomethyl (JB-647) derivatives linked at the 3 position. These "reversed" esters were generally not as potent as the diquaternarized esters of piperidinecarboxylic acid previously reported (1). JB-579,  $\beta$ -(3-diethylamino-1-propynyl)-N-methylpiperidine was the most active compound of a series of  $\beta$ -dialkylaminoalkynyl-N-methylpiperidines and was approximately six times as potent as the diethylamino-2-butyryl derivative (JB-631). The following compounds, not listed in Table I, were found to be relatively inactive: N'-methyl-3-piperidylmethyl N-methylnipecotate dihydro-

chloride (JB-559), N-methyl-3-piperidylmethyl N-methylnipecotate dimethobromide (JB-609), N-methyl-3-piperidylmethylaminopropionate dimethobromide (JB-593), N-methyl-3-piperidyl dimethylaminoacetate dimethobromide (JB-606), 3-dimethylaminopropyl N-methylnipecotate bimaleate (JB-648), and 1,5-bis-(diethylamino)-3-pentyl 1'-methylnipecotate trimethobromide (JB-652).

The administration of JB-654, 50 mg./Kg. (10 times the i. v. dose) by gastric intubation to anesthetized normotensive rats, produced a mean drop in blood pressure of 54% with a mean time to return to predrug level of one hundred and eighty minutes. Both the percent drop and duration of action were slightly greater than that produced by 5 mg./Kg., i. v. JB-654, 50 mg./Kg., administered via gastric intubation to unanesthetized rats produced a mean drop in blood pressure of 54% with a mean time to return to predrug levels of one hundred and seventy-two minutes. The maximum drop in blood pressure occurred between

TABLE I.—THE EVALUATION OF CERTAIN DIQUATERNARIZED PIPERIDINE DERIVATIVES ON THE BLOOD PRESSURE OF NORMOTENSIVE RATS<sup>a</sup>

Compound JB-	Dose, mg./Kg.	No of Rats	Drop in Blood Pressure, % ± S. D.	Mean Time to Return to Original Levels, Min ± S. D.	Rating <sup>b</sup>
608	2 0	6	47 7 ± 12 2	69.3 ± 18.0	++
643	5 0	4	51 9 ± 11.9	152 3 ± 52.2	++++
654	5 0	6	42 3 ± 7.5	156 0 ± 51 2	++++
592	25 0	4	19 0 ± 16 9	5 4 ± 2.6	+
607	10 0	4	48 8 ± 2 2	26 9 ± 7 5	+
647	5 0	8	41 9 ± 12 6	58 0 ± 19 1	++
642	5 0	6	44 1 ± 9 9	50.7 ± 17 5	++
646	10 0	4	32 4 ± 9 7	2 0 ± 0 2	+
641	20 0	4	46 1 ± 0 8	87 5 ± 19 2	+++
549	5 0	4	54 4 ± 10 7	108 0 ± 25 3	+++
552	20 0	4	51 5 ± 20 1	74 5 ± 17 9	++
579	10 0	4	46 9 ± 3 8	150 0 ± 48 5	++++
580	10 0	4	48 0 ± 6 7	38 8 ± 15 4	+
585	10 0	4	46 0 ± 17 0	18 8 ± 7 9	+
589	10 0	4	51 0 ± 1 7	42 5 ± 3 3	++
631	10 0	5	46 5 ± 9 2	25 0 ± 8 7	+
632	10 0	6	45 9 ± 8 2	18 7 ± 24 0	+
Hexamethonium	5 0	8	46 5 ± 17 9	30 0 ± 15 2	+
Pentolinium	1 0	11	48 7 ± 18 6	118 5 ± 40 3	+++
Chlorisondamine	0 5	14	44 6 ± 13 8	141 2 ± 51 6	++++

<sup>a</sup> Administered intravenously  
<sup>b</sup> Rating +, 0-10 minutes' duration, ++, 11-80 minutes, +++, 81-120 minutes, +++, 121+ minutes

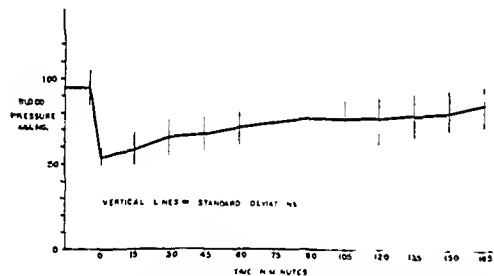


Fig. 2.—The effects of 5 mg/Kg. of JB-654, administered i. v., on the blood pressure of five normotensive albino rats

TABLE II.—THE EFFECTS OF CERTAIN HYPOTENSIVE AGENTS ON THE BLOOD PRESSURE OF NORMOTENSIVE DOGS<sup>a</sup>

Compound JB-	Dose, mg./Kg.	No of Dogs	Mean Drop in Blood Pressure, %	Time to Return to Original Levels, Min.
608	1 0	3	32	232+
608	2.0	1	58	510+
643	2.5	1	41	246
643	5 0	1	27	174
654	2.5	1	36	142
654	5 0	2	39	350
654	10.0	1	38	352
647	2.5	1	34	165
647	5.0	2	41	185
549	2.5	2	57	180
549	5.0	2	59	750+
552	25.0	4	64	398
579	10 0	1	56	450
580	10 0	1	30	120
589	1.0	1	53	25
589	10 0	1	45	143

<sup>a</sup> Administered intravenously

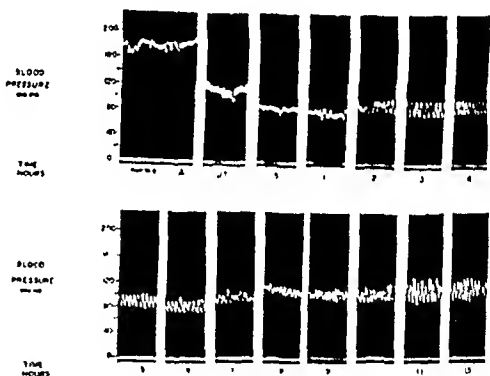


Fig. 3.—The effect of JB-643 on the blood pressure of a normotensive unanesthetized dog.

thirty and sixty minutes after the compound was administered.

**Hypotensive Effects in Dogs.**—The hypotensive activity of certain diquaternarized piperidine derivatives administered intravenously is summarized in Table II. The most active compounds were JB-549, JB-608, JB-579, JB-552, JB-654, and JB-643. The results observed in dogs were similar to those observed in rats in that five of the six most active compounds were either 3+ or 4+ in the rats evaluation studies. The only compound which demonstrated a significant difference in activity in the two species was JB-608 which produced marked hypotensive effects in four dogs but only elicited mild hypotensive responses in rats. Since JB-608 is the propyl analog of JB-591, a compound previously reported by Hudak, *et al.*, to possess excellent hypotensive activity (1), it was anticipated that this compound would also be a potent hypo-

tensive drug and it is most probable that the results obtained in the dog may be more significant than the effects of the compound in the rat

JB-643 and JB-654 produced marked hypotensive effects in unanesthetized dogs when administered via the oral route. JB-643 (100 mg/Kg) produced drops in blood pressure ranging between 34 and 55% with a mean duration of action in excess of eleven hours (Fig. 3). JB-654 (100 mg/Kg) produced depressor responses between 31 and 34% with a duration of action in excess of nineteen hours. Both compounds dilated the pupils and relaxed the nictitating membrane. The effects of JB-643 on the nictitating membrane were still evident sixty hours after the administration of the compound. The maximum hypotensive effects produced by both compounds occurred within forty minutes. The results of the oral studies with JB-643 and JB-654 indicate that slightly more than 10% of the compound is initially absorbed.

### SUMMARY

1. Twenty-two diquaternarized piperidine and related compounds were evaluated for their hypotensive activity.

2. 3-Morpholinopropyl N-methylpipercolinate dimethiodide (JB-643), 2-morpholinoethyl N-methylpipercolinate dimethobromide (JB-654),  $\beta$  - (3 - diethylamino - 1 - propynyl) - N - methylpiperidine dimethobromide (JB-579) were the most potent hypotensive compounds in this current series.

3. JB-643 and JB-654 exhibited marked hypotensive effects when administered via the oral route to unanesthetized dogs.

4. A method has been described by which direct, continuous blood pressure recordings can be obtained from unanesthetized dogs.

### REFERENCES

- (1) Hudak, W. J., Buckley, J. P., Schalit, F. M., DeFeo, J. J., and Reif, E. C., *THIS JOURNAL*, **46**, 595 (1957).
- (2) Buckley, J. P., Kelly, W. J., Hudak, W. J., and Bickerton, R. K., *Fed. Proc.* **18**, 373 (1959).
- (3) Biel, J. H., Sprengler, E. P., and Friedman, H. L., *J. Am. Chem. Soc.* **79**, 6184 (1957).
- (4) Bickerton, R. K., Jacquart, M. L., Kinnard, W. J., Bianculli, J. A., and Buckley, J. P., *THIS JOURNAL*, **49**, 183 (1960).
- (5) Still, J. W., Pradhans, S. N., and Whitcomb, E. R., *J. Appl. Physiol.* **8**, 875 (1956).

## Studies on *Vinca major* L. (*Apocynaceae*) I\*

### Isolation of Perivincine

By N. R. FARNSWORTH, F. J. DRAUST†, R. W. SAGER, and J. A. BIANCULLI

Perivincine has been isolated from the leaves and stems of *Vinca major* L. (*Apocynaceae*). The isolation and identification procedures are described, with the identity of perivincine being established by a mixed melting point with a known sample from *Vinca minor*. The optical rotation and pK determinations are reported for this alkaloid, and paper chromatograms were run with the  $R_f$  values determined. Preliminary screening of perivincine for hypotensive activity in anesthetized rats indicates that the alkaloid produces a rapid transient drop in carotid blood pressure.

PLANTS OF THE GENUS *Vinca* have been investigated extensively during the past few years with most of the studies being carried out in the search for new alkaloids with potential medicinal uses. Recently several investigators have reported that *Vinca* species possess possible activity in the treatment of hypertension (1),

leukemia (2, 3), and cancer (3). We have been interested in the alkaloids of *Vinca major* as a potential source of new medicinal agents. Seven alkaloids have been reported from *Vinca major*. They are reserpine (4), vincamajoridine (5), vincamajoreine (6), vineamajine (7), pubescine, vinine, and another crystalline alkaloid with a melting point of 194–195° (8).<sup>1</sup> Vinine has been reported to elicit a pronounced and prolonged blood pressure drop in experimental animals (8), whereas reserpinine has been shown to produce

\* Received October 28, 1959, from the University of Pittsburgh, School of Pharmacy, Pittsburgh 13, Pa.  
Abstracted in part from a dissertation submitted to the Graduate Faculty of the University of Pittsburgh, School of Pharmacy, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

This study was supported in part by a grant from the Health Research and Services Foundation, Pittsburgh 19, Pa.

† Present address: University of Pittsburgh, School of Dentistry, Pittsburgh 13, Pa.

<sup>1</sup> Pubescine, vinine, and the alkaloid with a melting point of 194–195° were isolated from *Vinca pubescens*, which is considered identical to *Vinca major* var. *major*.

only a transient blood pressure drop (9) The total alkaloids of *Vinca major* have been shown by Quevauviller, *et al*, to act as ganglioplegics and sympatholytics but do not exert more than a transient drop in blood pressure (10) None of the remaining alkaloids isolated from this plant have been reported to produce any biological activity

In this paper we wish to report the isolation and properties of perivincine from *Vinca major* This alkaloid has previously been reported only from *Vinca minor* (11)

## EXPERIMENTAL

**Extraction of *Vinca major* Leaves and Stems.**—The air-dried ground leaves and stems of *V. major* were packed in a large capacity Soxhlet-type extraction apparatus and extracted with a mixture of 20 volumes of 95% ethanol and 1 volume of 100% methanol for twenty-four hours, or until the leaves and stems were free of alkaloids The percolate was allowed to cool and was filtered to remove a small amount of nonalkaloidal brown residue The mixed alcohol-soluble extract was concentrated at 55° under reduced pressure to 10% of its original volume utilizing a continuous model flash evaporator<sup>2</sup> This concentrate was placed in a refrigerator at 5° overnight and again filtered to remove nonalkaloidal residue<sup>3</sup>

**Isolation of the Total Alkaloids.**—One thousand grams of extract was treated with 2,000 ml of 5% acetic acid which produced a large amount of black resinous material The mixture was shaken for one hour in a refrigerated room at 5° and then extracted with four separate 500-ml portions of *n*-hexane at 5° or until the *n*-hexane was no more than faint yellow in color The *n*-hexane layers were separated, pooled, and the *n*-hexane was removed by distillation The dark green residue, after drying to constant weight at 60°, represented 5.3% of the air-dried leaves and stems The *n*-hexane extract was free of alkaloids

The aqueous acidic extract was kept in a refrigerated room at 5° for twenty-four hours and freed from a small amount of brown residue by filtration This residue was free of alkaloids and was discarded The filtrate was concentrated at 55° under reduced pressure to one-third the original volume utilizing a continuous model flash evaporator, then treated with 28% ammonium hydroxide at 5° to a pH of 10 with constant stirring, and the alkaloidal precipitate was removed by filtration The residue was washed with several portions of distilled water and dried at 60° for twenty-four hours The filtrate (filtrate A) which was positive for alkaloids was extracted with chloroform The chloroform extract was dried with anhydrous sodium sulfate, filtered, and the chloroform was removed by distillation After drying under

reduced pressure to constant weight it represented 0.19% of the air-dried leaves and stems

The alkaloidal residue as removed from the alkaline solution by filtration was very tarry. To further purify this material the residue was taken up in 400 ml of hot 5% acetic acid, filtered (filtrate B), and reprecipitated with 28% ammonium hydroxide The alkaloidal precipitate, after washing with distilled water and drying to constant weight, was converted from a black tarry mass to a gray powder which represented 0.12% of the air-dried leaves and stems The filtrate (filtrate C) was extracted at room temperature with chloroform as described earlier to yield an additional 0.05% of material The total yield of crude alkaloid was 0.36%

**Isolation and Properties of Perivincine.**—The total alkaloids removed by filtration and by extraction of filtrates A and C with chloroform were combined In a typical experiment, 22 Gm of the total alkaloids were placed in a suitable flask and refluxed with 200 ml of benzene for two hours The benzene solution was cooled to room temperature, and the benzene-soluble material removed from the flask This procedure was repeated with 200-ml followed by 100-ml quantities of benzene, and the benzene extracts were combined and slowly added to 1,000 ml of petroleum ether (b.p. 30–60°) with constant stirring A yellow precipitate formed which turned dark brown on standing Petroleum ether was added until precipitation was complete The mixture was filtered and the filtrate was concentrated on a water bath to a volume of approximately 15 ml The concentrate was placed in a refrigerator at 5° for twenty-four hours, at which time small rosette crystals were embedded in a reddish oil Fifteen milliliters of absolute ether was added and the mixture was stirred until all of the oil and the crystals were dissolved The solution was allowed to evaporate spontaneously in a dark place, during which time copious amounts of fine needle-like crystals began to form These were removed by filtration and washed with several portions of ice-cold absolute ethanol The crude perivincine crystals were dried, weighed, and found to represent from 1.6 to 1.8% of the total alkaloids Purification of the crude perivincine was effected by several recrystallizations from hot 95% ethanol to a constant melting point

**Anal** <sup>4</sup>—Calcd. for  $C_{22}H_{25}N_2O_4$ : C, 68.75; H, 7.35, N, 7.29 Found: C, 69.54, H, 7.42; N, 7.37

The melting point of perivincine determined in a capillary tube (uncorrected), was 199.5–200° (decompn) and there was no depression when mixed with an authentic specimen of perivincine isolated from *Vinca minor*<sup>5</sup>

**Optical Rotation.**—The optical rotation of perivincine was determined by measurement in a Zeiss-Winkel polarimeter with a sample tube length of 1.15 cm The optical rotation for perivincine was found to be  $[\alpha]_D^{25} = -61 \pm 2^\circ$  (pyridine,  $c = 0.5$ )

<sup>2</sup> Flash evaporator, continuous model, Laboratory Glass and Instruments Corp., New York 31, N. Y.

<sup>3</sup> An extract prepared in this manner as well as the leaves and stems for the extraction were generously supplied by the S. B. Penick & Co., Inc., 100 Church Street, New York 8, N. Y. The extract as supplied was calculated to be a 44.89% (w/w) concentrate

<sup>4</sup> Microanalysis of perivincine was conducted by the Analytical Chemistry Section, Research Services of the Mellon Institute, 4400 Fifth Ave., Pittsburgh 13, Pa.

<sup>5</sup> An authentic specimen of perivincine was obtained through the courtesy of Dr. Nathan Rubin, Department of Chemistry, Philadelphia College of Pharmacy and Science, Philadelphia, Pa.

**pK Determination.**—The pK of perivincine was determined by potentiometric titration of the alkaloids using 75%(v/v) dimethylformamide (Eastman) as the solvent. A Beckman model G pH meter equipped with calomel and glass electrodes was employed for the titration. From the titration data the pK<sub>a</sub> of perivincine was calculated to be 8.20.

**Ultraviolet and Infrared Spectra.**—The ultraviolet absorption spectrum of perivincine was determined at a concentration of 0.01 mg/ml in 95% ethanol using a Beckman ratio recording spectrophotometer model DK-2. In addition, the infrared absorption spectrum was determined in a Nujol mull, using a Beckman spectrophotometer model IR-4.<sup>6</sup> Both the ultraviolet and infrared data for the perivincine isolated in this study corresponded to the data recorded for perivincine isolated from *Vinca minor* by Scheindlin and Rubin (11).

**Screening of Perivincine for Hypotensive Activity.**—Perivincine was screened for hypotensive activity in normotensive as well as in hypertensive rats. Albino Wistar rats were made hypertensive by the standard procedure of unilateral nephrectomy one week after a figure-of-eight ligature was placed on the contralateral kidney. The rats were fed a normal diet and administered 0.25 ml of a desoxycorticosterone trimethylacetate suspension<sup>7</sup> (25 mg./ml.) subcutaneously once a week for ten weeks. A 1% normal saline solution was given in place of drinking water once weekly. At the end of this period of time the rats were anesthetized with urethan and prepared for direct carotid blood pressure recordings according to the method described by Hudak, *et al* (12).<sup>8</sup>

Perivincine was dissolved in Serpasil placebo solvent<sup>9</sup> (7.5 mg./ml.) and the solution was slowly administered into a femoral vein. Equivalent volumes of solvent were administered intravenously prior to the perivincine solution. A slight transient lowering of blood pressure was noted. Single doses of perivincine ranging from 5 to 20 mg./Kg. were then administered to both normotensive as well as hypertensive rats.

In the rats studied, perivincine produced a rapid transient drop in blood pressure of from 27 to 40%. The return to normal was usually within twenty to thirty minutes. This preliminary screening of perivincine for hypotensive activity indicated that the alkaloid is not a potentially good hypotensive agent.

**Paper Chromatography of Perivincine.**—The method described by Korzun, *et al* (13), for the paper chromatographic evaluation of *Rauwolfia* species was modified for the evaluation of *Vinca major* alkaloids. In this method, formamide-saturated paper was used with a mobile phase

consisting of equal parts of benzene and cyclohexane, saturated with formamide. Since formamide, as obtained commercially, was found to vary in pH from 10 to 11.7 it was necessary to standardize it in order to obtain reproducible results. This was done by adjusting the formamide with formic acid to various pH levels. It was found that the best resolution of alkaloidal spots was obtained at formamide pH values of 5.2 and 8.0.

Perivincine, dissolved in chloroform, and chromatographed according to this modified method of Korzun, *et al* (13), exhibited *R<sub>f</sub>* values of 0.08 with formamide adjusted to a pH of 5.2 and *R<sub>f</sub>* 0.17 with formamide adjusted to a pH of 8.0 using a modified Dragendorff's reagent (14) and long wave ultraviolet light to reveal the alkaloidal spots.

The preliminary fractionation of the total alkaloids on alumina columns with subsequent paper chromatographic evaluation of each fraction indicates the presence of at least 37 alkaloids in this plant and the results strongly suggest the presence of tetrahydroalstonine, sarpagine, vincamine, and serpentine. The exact procedure and experimental data for the chromatographic separation of the total alkaloids of *Vinca major* will be published at a later date.

## SUMMARY

1. Perivincine has been isolated and identified from the leaves of *Vinca major* L. (*Apocynaceae*).
2. Several analytical constants for this alkaloid have been verified and the optical rotation and pK<sub>a</sub> determinations have been established.
3. The *R<sub>f</sub>* value of perivincine has been reported in a modified chromatographic procedure at 2 pH values.
4. Preliminary screening of perivincine in anesthetized normotensive and hypertensive rats indicated a rapid, transient drop in blood pressure ranging from 27 to 40 per cent with a return to normal within twenty or thirty minutes.

## REFERENCES

- (1) Szczeklik, E., Hano, J., Bozdamkova, B., and Maj, J., *Polski Tygodnik Lekarski*, 12, 121(1957).
- (2) Svoboda, G. H., *THIS JOURNAL*, 47, 834(1958).
- (3) Noble, R. L., Beer, C. T., and Cutts, J. H., *Ann N Y Acad Sci*, 1958, 893.
- (4) Janot, M. M., and LeMen, J., *Compt rend.*, 238, 2550(1954).
- (5) Janot, M. M., and LeMen, J., *ibid*, 240, 909(1955).
- (6) Janot, M. M., and LeMen, J., *Ann pharm franc*, 13, 325(1955).
- (7) Janot, M. M., and LeMen, J., *Compt rend.*, 241, 767(1955).
- (8) Oreschoff, A., Gurewitsch, H., Norkina, S., and Prein, N., *Arch Pharm*, 272, 70(1934).
- (9) Bein, H. J., *Pharmacol Rev*, 8, 448(1956).
- (10) Quevauxviller, A., LeMen, J., and Janot, M. M., *Ann pharm franc*, 13, 328(1955).
- (11) Scheindlin, S., and Rubin, N., *THIS JOURNAL*, 44, 330(1955).
- (12) Hudak, W. J., Buckley, J. P., Schalit, F. M., DeFeo, J. J., and Reis, E. C., *ibid*, 46, 595(1957).
- (13) Korzun, B. P., St. Andre, A. F., and Ulshafer, P. R., *ibid*, 46, 720(1957).
- (14) Munier, R., and Macheboeuf, M., *Bull soc. chim biol*, 31, 1144(1949).

<sup>6</sup> Grateful acknowledgment is made to Dr. Hans Noll, Department of Microbiology, School of Medicine, University of Pittsburgh, for performing the infrared study of perivincine.

<sup>7</sup> Generously supplied as Percorten Trimethylacetate intramuscular repository by the Ciba Pharmaceutical Products Inc., Summit, N. J.

<sup>8</sup> The authors wish to thank Mr. Robert K. Bickerton for his assistance in performing the hypotensive screening.

<sup>9</sup> Generously supplied by the Ciba Pharmaceutical Products Inc., Summit, N. J.

# A Kinetic Study of Glucose Degradation in Acid Solution\*

By KENNETH R. HEIMLICH† and ALFRED N. MARTIN

The degradation of glucose in acid solution was carried out at various temperatures. The first-order rate constants for the decomposition reaction, obtained from the initial rate of formation of 5-hydroxymethylfurfural (5-HMF), were consistently higher than those calculated from glucose depletion measurements over long periods of time at temperatures above 100°. At 100° the rate of formation of 5-HMF increased with time for the early portion of the break-down. The rate constant did not coincide with the Arrhenius plot for higher temperatures. This indicates the possibility of a change of mechanism at lower temperatures. The rate data was fitted to kinetic expressions for first-order consecutive reactions. Agreement between calculated and experimental values indicates that the reaction proceeds by the step-wise mechanism. Data obtained from degradation in varying acid concentrations show that the rate constant does not vary linearly with normality. Decomposition in dioxane-water mixtures indicates that the logarithm of the rate constant varies linearly with the reciprocal of the dielectric constant.

A LARGE PORTION of the glucose employed in the pharmaceutical industry is used for the preparation of intravenous solutions. Upon storage these solutions develop a faint straw color, and this instability creates a problem when large quantities of the material are stockpiled for use in times of emergency. Webb, *et al.* (1), carried out a preliminary investigation on the degradation of glucose as part of a project for the Armed Forces. The present study is a continuation of this work with emphasis on the kinetics of the decomposition.

The general scheme of glucose degradation in acid solution has been given by Wolfram, Schuetz, and Cavalieri (2). The decomposition involves the conversion of glucose to 5-hydroxymethylfurfural (abbreviated in this report as 5-HMF) with the subsequent breakdown of this material to formic and levulinic acids and a colored material. It has been indicated by Scallet and Gardner (3) and Joslyn (4) that the color which develops is due to the polymerization of 5-HMF. Saeman (5) studied the breakdown under severe conditions of temperature and acid concentration and also in atmospheres of air, oxygen, and carbon dioxide; he found the depletion to be first order and not affected by the different atmospheres.

Because of the slowness of glucose breakdown at temperatures of 100° and lower, many investigators have used the formation of 5-HMF, which is determined spectrophotometrically, as a

criterion for the degradation. Singh, Cantor, and Dean (6) reported that the rate of formation of 5-HMF was indicative of the amount of glucose decomposed. Webb (1) also used the formation of 5-HMF to study the breakdown under varying conditions of catalysis, concentration of glucose, and temperature.

In addition to the degradation to 5-HMF, glucose may undergo a so-called reversion reaction. Many investigators (7-12) have shown this reaction to be manifested as a reversible polymerization condensation to higher saccharides and also the formation of anhydro sugars. It has been stated by Fetzner, *et al.* (9), that the literature on this process is extensive and confusing. Under conditions of low glucose and low acid concentrations the amount of products of this reaction is small.

## EXPERIMENTAL

**Materials.**—Mallinckrodt analytical reagent, anhydrous glucose was used for all tests, and the solutions were prepared with deionized, distilled water. The 5-HMF was obtained from the Bios Laboratories (17 W. 60th St., New York, N. Y.). It was purified by vacuum distillation at a reduced pressure of 2 mm. Hg. The dioxane used was Eastman's analytical grade, and was purified according to the method given by Kraus and Vinge (13). All other chemicals used were of the highest purity available, usually analytical reagent or reagent grade. The decomposition of the solutions was carried out in 5-ml. Tuf-Top Neutraglas ampuls obtained from the Kimble Glass Co., Toledo, Ohio.

**Apparatus.**—The constant temperature source was a Bayley model 118 bath (Bayley Instrument Co., Danville, Calif.). The bath has a stated precision of  $\pm 0.001^\circ$ . The 5-HMF concentrations were determined on a Beckman DU spectrophotometer with the ultraviolet attachment. The solutions

\* Received August 21, 1959, from Purdue University, School of Pharmacy, Lafayette, Ind.

This work was supported by a grant from the U. S. Medical Procurement Agency.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

† Present address: Smith Kline and French Laboratories, Philadelphia, Pa.

were read in fused silica cells. The pH determinations were made on a Beckman model G pH meter.

**Assay.**—The degradation of the glucose solutions was followed either by the formation of 5-HMF or the depletion of glucose, and in some cases both methods were employed on the same solution. The determination of 5-HMF was done spectrophotometrically. The solutions were filtered to remove any insoluble humus material, diluted, and read against a glucose blank of the proper concentration at a wavelength of 284  $m\mu$ . The absorption readings were converted to moles per liter by the expression

$$C = A \times 6.298 \times 10^{-6} \quad (\text{Eq. 1})$$

where  $A$  is the absorbance of the solution. This equation was obtained from the standard curve of a Beer plot.

The glucose was determined by an iodometric method first used by Romijn (14) and further tested by Cajori (15). The method consists of reacting the solutions with a measured excess of iodine and titrating the residual iodine with standard thiosulfate solution. The equation for converting to the amount of glucose present is

$$\text{moles/liter of glucose} = \frac{(R - S) \times N \times 0.1}{2} \quad (\text{Eq. 2})$$

where  $N$  is the normality of the thiosulfate solution,  $R$  is the milliliters of thiosulfate required to react with an iodine blank, and  $S$  the amount required to react with the sample. The accuracy of the method was determined to be from 1 to 3% in the presence of decomposition products of the reaction.

## RESULTS AND DISCUSSION

The decomposition of 0.056 molar glucose solutions in 0.35  $N$  hydrochloric acid was followed by glucose depletion and by 5-HMF formation from 100 to 150°. Plots of the logarithm of the amount of glucose remaining against time produced good linearity which indicates a first-order decomposition. The rate constants were calculated from

$$k_t = (2.303/t) \log (A_0/A) \quad (\text{Eq. 3})$$

where  $A$  is the concentration of glucose in moles/liter at any time,  $t$  is the time in hours,  $A_0$  is the original concentration of glucose in moles/liter, and  $k_t$  is the first-order rate constant having the unit of reciprocal time. These values are given in Table I.

TABLE I.—FIRST-ORDER RATE CONSTANTS FOR GLUCOSE BREAKDOWN IN 0.35  $N$  HYDROCHLORIC ACID

Temperature, °C	Rate Constant, $k_t$ hr <sup>-1</sup>
100	0.0012
110	0.0040
120	0.0120
130	0.0267
140	0.0628
150	0.1693

Under the conditions used in this study the induction type curve for 5-HMF formation reported by Webb, *et al.* (1), was evidenced only at 100°

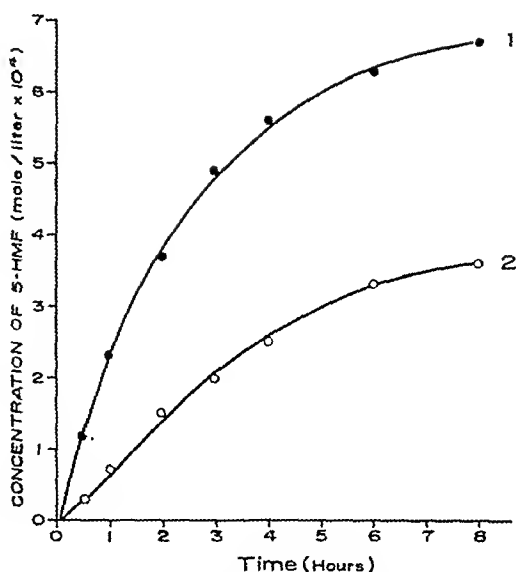


Fig. 1.—The formation of 5-hydroxymethylfurfural at 110° (curve 1) and at 100° (curve 2).

The changing nature of the curve with time and temperature is shown in Fig. 1. Rate constants based on 5-HMF formation were obtained from plots of this nature by calculating the initial slope,  $(dB/dt)_t$ . These values were obtained by placing a mirror on the curve perpendicular to the plane of the paper and adjusting it until the image of the curve produced a smooth, continuous arc with the curve itself. The slope of a line perpendicular to the mirror as read from the plot at zero time yielded the desired value. Assuming that the formation of 5-HMF is a measure of glucose depletion, and disregarding the possible consecutive steps of the reaction at this point (*cf.* Eq. 6) the following expression may be written

$$(dB/dt)_t = k_t A \quad (\text{Eq. 4})$$

where  $(dB/dt)_t$  is the initial slope of the formation curve,  $k_t$  is the first-order rate constant, and  $A$  is the concentration of glucose. In order to obtain the rate constant the equation is rearranged to

$$k_t = (dB/dt)_t / A \quad (\text{Eq. 5})$$

The subscript  $t$  is used in the  $k_t$  since the rate constant is based on the initial rate. The values of  $k_t$  from 100 to 140° are given in Table II.

A comparison of Tables I and II shows that the values of  $k_t$  are consistently smaller than the values of  $k_t$  except at 100°. It is important to point out

TABLE II.—VALUES OF  $k_t$  OBTAINED FROM 5-HMF FORMATION FOR GLUCOSE BREAKDOWN IN 0.35  $N$  HYDROCHLORIC ACID

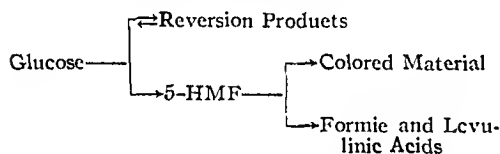
Temperature, °C	$k_t$ , hr <sup>-1</sup>
100	0.0009
110	0.0054
120	0.0179
130	0.0330
140	0.1063



this difference in the rate constants obtained by the two different methods, since glucose decomposition studies in the literature have been based on one or the other of these criteria. Some authors have calculated the rate constant from the breakdown at some arbitrary time rather than initially. The present study shows that this procedure is unsatisfactory because of the changing slope of the 5-HMF curve. Since the initial rate is the velocity of the reaction before any interference from products can occur,  $k_i$  may be accepted as the more significant quantity. The  $k_i$  values, obtained from the integrated equation, are based on the degradation reaction over a long period of time. Even though the reaction follows the integral order with respect to the method of integration it is possible that products of the reaction are interfering, giving  $k_i$  values which are lower than the  $k_i$  values based on the initial formation of the 5-HMF. Laidler (16) has also pointed to the possible discrepancy between these two rate constants and has recommended the use of  $k_i$  for complex reactions such as involved in the present study.

The logarithm of the rate constants,  $k_i$  and  $k_t$ , were plotted against the reciprocal of the absolute temperature according to the Arrhenius equation and are shown in Fig. 2. The activation energy calculated from  $k_i$  values was 31.8 Kcal/mole which compares favorably to that of 31.2 Kcal/mole obtained from  $k_t$  values.

**Application of the Rate Data to a Kinetic Expression.**—From the literature (2-4, 11, 17) and from our own observation, the degradation of glucose in acid solution may be represented by the scheme



The reversion reaction is reversible, and at low glucose and acid concentrations the formation of reversion products is slight and may be neglected here. Due to the indefinite nature of the 5-HMF breakdown products, the expression for the degradation of glucose may be written simply as



where  $a$  is glucose,  $b$  is 5-HMF,  $c$  is the breakdown products of 5-HMF, and  $k_1$  and  $k_2$  are the first-order rate constants. A consecutive reaction such as this was studied by Harecourt and Esson (18), and the mathematical expressions were derived by Esson (19). If  $A_0$  is the initial concentration of  $a$ , and  $A$ ,  $B$ , and  $C$  are the concentrations of  $a$ ,  $b$ , and  $c$ , respectively, at any time,  $t$ , then

$$A_0 = A + B + C \quad (\text{Eq. 7})$$

The rate of disappearance of  $a$  is given at any instant by

$$-dA/dt = k_1 A \quad (\text{Eq. 8})$$

which yields upon integration

$$A = A_0 e^{-k_1 t} \quad (\text{Eq. 9})$$

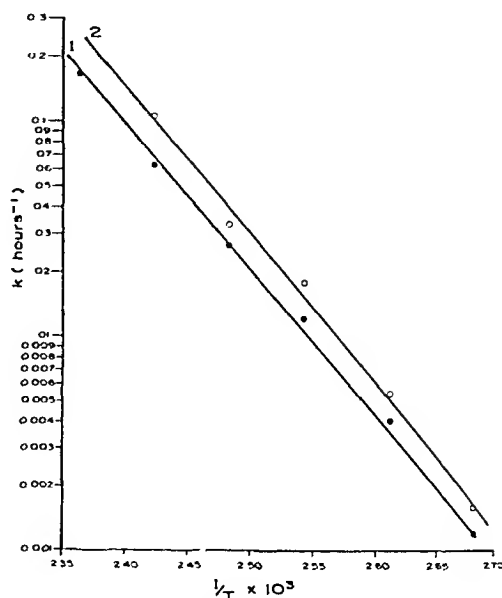


Fig. 2.—Temperature dependence of  $k_1$ , the first-order rate constant based on glucose depletion (curve 1), and  $k_t$ , the first-order rate constant based on the initial formation of 5-hydroxymethylfurfural (curve 2).

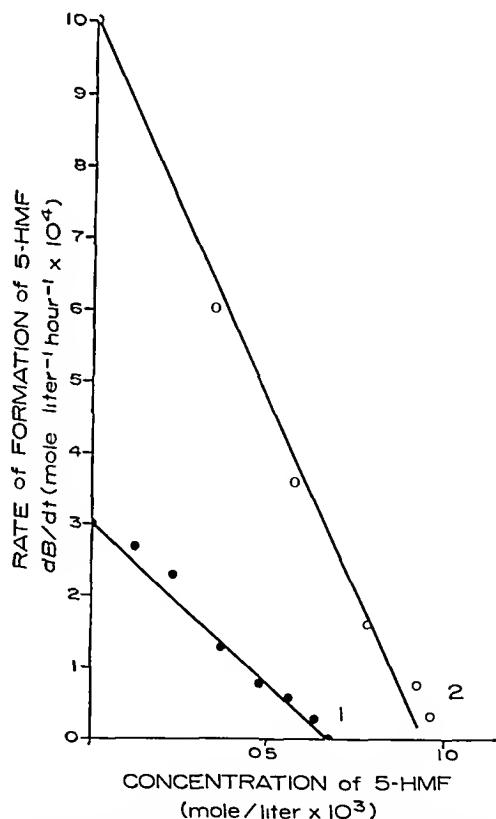


Fig. 3.—The rate of formation of 5-hydroxymethylfurfural versus the concentration of 5-hydroxymethylfurfural at 110° (curve 1) and 120° (curve 2).

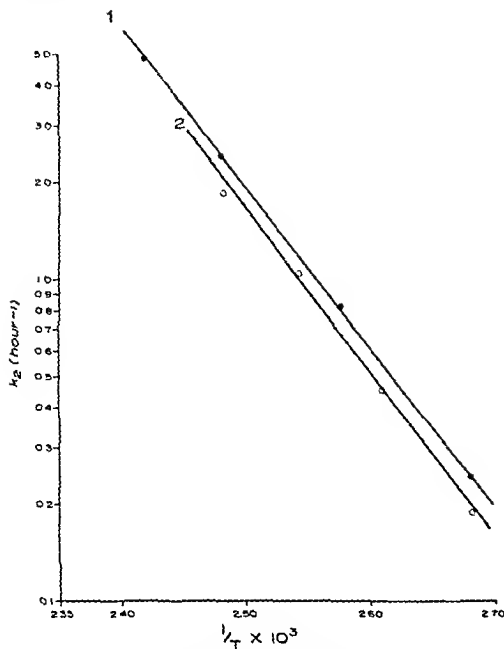


Fig. 4.—Temperature dependence of the experimental rate constant (curve 1) and the calculated rate constant (curve 2) for 5-hydroxymethylfurfural degradation.

The rate at which the concentration of  $b$  increases is given by

$$dB/dt = k_1A - k_2B \quad (\text{Eq. 10})$$

which upon integration and substitution of  $A$  from Eq. 9 becomes

$$B = \frac{A_0 k_1}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}) \quad (\text{Eq. 11})$$

The formation of  $c$  is given by the expression

$$dC/dt = k_2 B \quad (\text{Eq. 12})$$

Upon integration and the substitution of  $B$  from Eq. 11 the following is obtained

$$C = A_0 \left[ 1 + \frac{1}{k_1 - k_2} (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \right] \quad (\text{Eq. 13})$$

The value of  $C$  may also be obtained from Eq. 7 by difference.

The data can be fitted to the kinetic expression by calculating the values of  $k_2$  by a graphical method employing Eq. 10. The left hand side of the equation represents the rate at which 5-HMF is forming at any time,  $t$ . The values of  $dB/dt$  were obtained by taking tangents from the plot of  $B$  versus  $t$  using a mirror as described earlier. These values of  $dB/dt$  were plotted against  $B$ . The result expected is a straight line plot, the slope of which is  $-k_2$  and whose intercept is  $k_1 A$ , where  $k_1$  is used instead of  $k_1$  for the reason previously given. Plots of this nature for 110 and 120° are shown in Fig. 3. Good linearity was found for the earlier portions of the breakdown in each case. The slopes of the plots

TABLE III.—A COMPARISON OF CALCULATED AND EXPERIMENTAL VALUES OF  $k_2$

Temperature, °C.	$k_2$ Hr. <sup>-1</sup> , Calcd.	$k_2$ Hr. <sup>-1</sup> , Exptl.
100	0.1875	0.2434
110	0.4533	0.5450
120	1.0500	1.2100
130	1.8400	2.4048

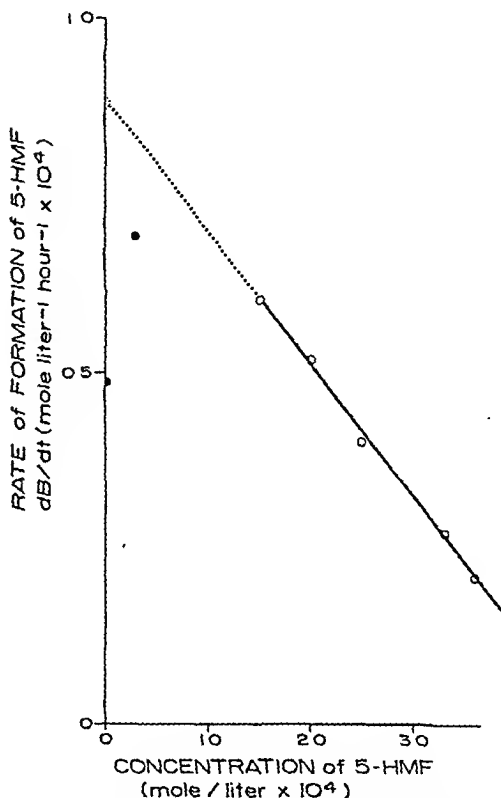


Fig. 5.—The rate of formation of 5-hydroxymethylfurfural versus the concentration of 5-hydroxymethylfurfural at 100°. Solid points show initial increase in rate at low concentrations. Extrapolation yields rate consistent with higher temperatures.

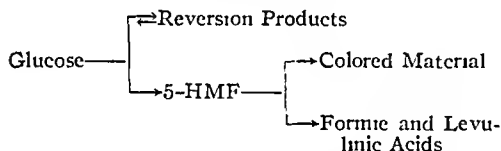
were taken to obtain the values of  $k_2$ . A comparison of  $k_2$  values calculated in this manner using Eq. 10, and  $k_2$  values obtained experimentally from the degradation of pure 5-HMF in the same acid concentration, is shown in Table III. The activation energies were obtained from the Arrhenius plots for each set of constants shown in Fig. 4. The activation energy obtained from the calculated values of  $k_2$  was 23.4 Kcal./mole while that from the experimental values of  $k_2$  was 22.7 Kcal./mole.

A plot of  $dB/dt$  (the rate of formation of 5-HMF) versus  $B$  (the concentration of 5-HMF) at 100° is shown in Fig. 5. The first 3 points on the plot indicate the increasing value of  $dB/dt$  as depicted by the increase in the slope of the initial portion of curve 2 in Fig. 1. The extrapolation of the initial portion of the curve in Fig. 5 to  $B = 0$  yields a value of  $k_1 A$  according to Eq. 10. The value obtained

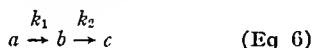
this difference in the rate constants obtained by the two different methods, since glucose decomposition studies in the literature have been based on one or the other of these criteria. Some authors have calculated the rate constant from the breakdown at some arbitrary time rather than initially. The present study shows that this procedure is unsatisfactory because of the changing slope of the 5-HMF curve. Since the initial rate is the velocity of the reaction before any interference from products can occur,  $k_1$  may be accepted as the more significant quantity. The  $k_1$  values, obtained from the integrated equation, are based on the degradation reaction over a long period of time. Even though the reaction follows the integral order with respect to the method of integration it is possible that products of the reaction are interfering, giving  $k_1$  values which are lower than the  $k_1$  values based on the initial formation of the 5-HMF. Laidler (16) has also pointed to the possible discrepancy between these two rate constants and has recommended the use of  $k_1$  for complex reactions such as involved in the present study.

The logarithm of the rate constants,  $k_1$  and  $k_t$ , were plotted against the reciprocal of the absolute temperature according to the Arrhenius equation and are shown in Fig 2. The activation energy calculated from  $k_t$  values was 31.8 Kcal/mole which compares favorably to that of 31.2 Kcal/mole obtained from  $k_1$  values.

**Application of the Rate Data to a Kinetic Expression.**—From the literature (2-4, 11, 17) and from our own observation, the degradation of glucose in acid solution may be represented by the scheme



The reversion reaction is reversible, and at low glucose and acid concentrations the formation of reversion products is slight and may be neglected here. Due to the indefinite nature of the 5-HMF breakdown products, the expression for the degradation of glucose may be written simply as



where  $a$  is glucose,  $b$  is 5-HMF,  $c$  is the breakdown products of 5-HMF, and  $k_1$  and  $k_2$  are the first-order rate constants. A consecutive reaction such as this was studied by Harcourt and Esson (18), and the mathematical expressions were derived by Esson (19). If  $A_0$  is the initial concentration of  $a$ , and  $A$ ,  $B$ , and  $C$  are the concentrations of  $a$ ,  $b$ , and  $c$ , respectively, at any time,  $t$ , then

$$A_0 = A + B + C \quad (\text{Eq. 7})$$

The rate of disappearance of  $a$  is given at any instant by

$$-dA/dt = k_1 A \quad (\text{Eq 8})$$

which yields upon integration

$$A = A_0 e^{-k_1 t} \quad (\text{Eq 9})$$

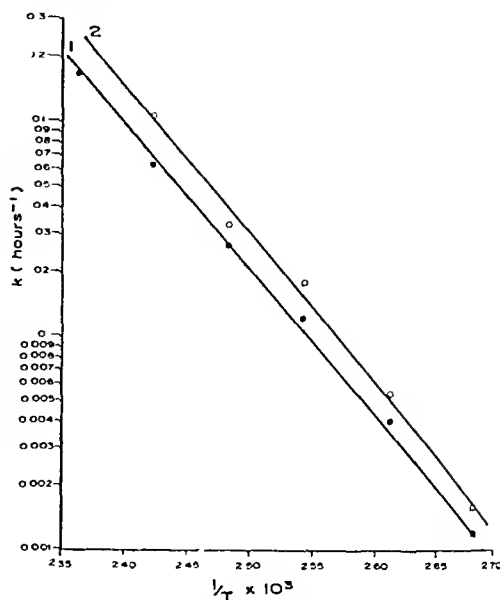


Fig 2—Temperature dependence of  $k_1$ , the first-order rate constant based on glucose depletion (curve 1), and  $k_t$ , the first-order rate constant based on the initial formation of 5-hydroxymethylfurfural (curve 2).

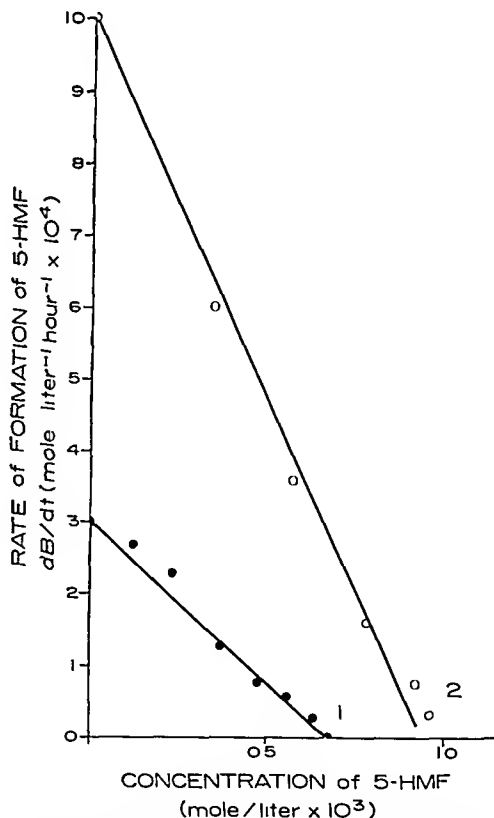


Fig 3—The rate of formation of 5-hydroxymethylfurfural versus the concentration of 5-hydroxymethylfurfural at 110° (curve 1) and 120° (curve 2).

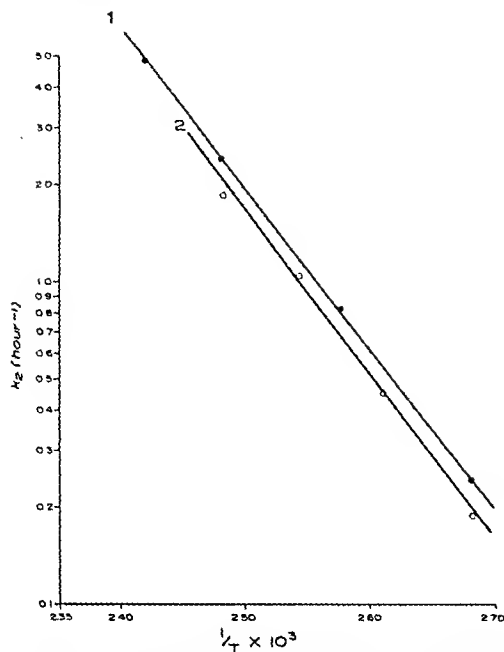


Fig. 4.—Temperature dependence of the experimental rate constant (curve 1) and the calculated rate constant (curve 2) for 5-hydroxymethylfurfural degradation.

The rate at which the concentration of  $b$  increases is given by

$$dB/dt = k_1A - k_2B \quad (\text{Eq. 10})$$

which upon integration and substitution of  $A$  from Eq. 9 becomes

$$B = \frac{A_0 k_1}{k_2 - k_1} (e^{-k_1 t} - e^{-k_2 t}) \quad (\text{Eq. 11})$$

The formation of  $c$  is given by the expression

$$dC/dt = k_2 B \quad (\text{Eq. 12})$$

Upon integration and the substitution of  $B$  from Eq. 11 the following is obtained

$$C = A_0 \left[ 1 + \frac{1}{k_2 - k_1} (k_2 e^{-k_1 t} - k_1 e^{-k_2 t}) \right] \quad (\text{Eq. 13})$$

The value of  $C$  may also be obtained from Eq. 7 by difference.

The data can be fitted to the kinetic expression by calculating the values of  $k_2$  by a graphical method employing Eq. 10. The left hand side of the equation represents the rate at which 5-HMF is forming at any time,  $t$ . The values of  $dB/dt$  were obtained by taking tangents from the plot of  $B$  versus  $t$  using a mirror as described earlier. These values of  $dB/dt$  were plotted against  $B$ . The result expected is a straight line plot, the slope of which is  $-k_2$  and whose intercept is  $k_1 A$ , where  $k_1$  is used instead of  $k_2$  for the reason previously given. Plots of this nature for 110 and 120° are shown in Fig. 3. Good linearity was found for the earlier portions of the breakdown in each case. The slopes of the plots

TABLE III.—A COMPARISON OF CALCULATED AND EXPERIMENTAL VALUES OF  $k_2$

Temperature, °C.	$k_2$ Hr. <sup>-1</sup> , Calcd.	$k_2$ Hr. <sup>-1</sup> , Exptl.
100	0.1875	0.2434
110	0.4533	0.5450
120	1.0500	1.2100
130	1.8400	2.4048

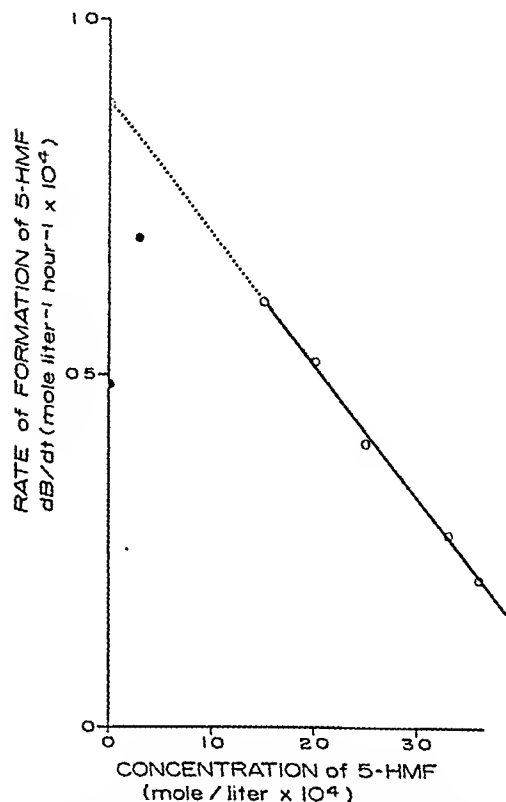


Fig. 5.—The rate of formation of 5-hydroxymethylfurfural versus the concentration of 5-hydroxymethylfurfural at 100°. Solid points show initial increase in rate at low concentrations. Extrapolation yields rate consistent with higher temperatures.

were taken to obtain the values of  $k_2$ . A comparison of  $k_2$  values calculated in this manner using Eq. 10, and  $k_2$  values obtained experimentally from the degradation of pure 5-HMF in the same acid concentration, is shown in Table III. The activation energies were obtained from the Arrhenius plots for each set of constants shown in Fig. 4. The activation energy obtained from the calculated values of  $k_2$  was 23.4 Kcal./mole while that from the experimental values of  $k_2$  was 22.7 Kcal./mole.

A plot of  $dB/dt$  (the rate of formation of 5-HMF) versus  $B$  (the concentration of 5-HMF) at 100° is shown in Fig. 5. The first 3 points on the plot indicate the increasing value of  $dB/dt$  as depicted by the increase in the slope of the initial portion of curve 2 in Fig. 1. The extrapolation of the linear portion of the curve in Fig. 5 to  $B = 0$  yields a value of  $k_1 A$  according to Eq. 10. The value obtained by

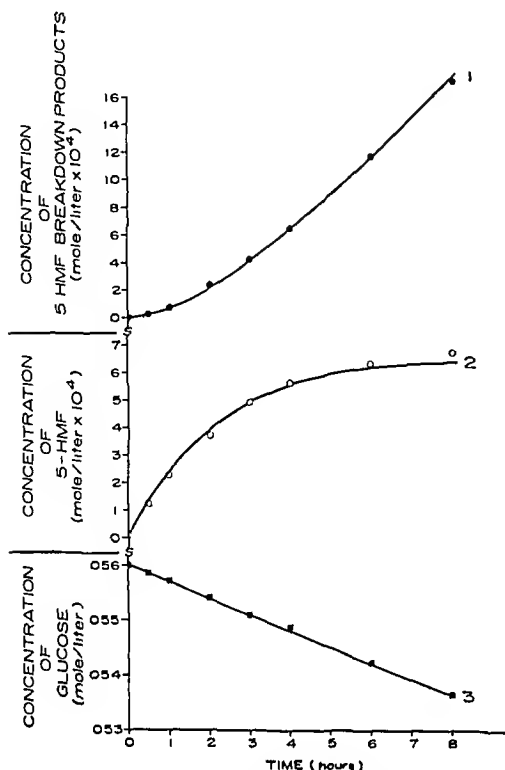


Fig 6—Concentration of 5 hydroxymethyl furfural breakdown products (curve 1), 5 hydroxymethylfurfural (curve 2), and glucose (curve 3), at various times at  $110^{\circ}$

this method for  $k_t$  was  $0.0016 \text{ hr}^{-1}$  as compared to  $0.0009 \text{ hr}^{-1}$  given in Table II. The former value is consistent with the Arrhenius plot for  $k_t$  values at higher temperatures given in Fig 2 (curve 2). Since the later value is not consistent with the plot, the possibility exists that at  $100^{\circ}$  and below the formation of 5-HMF may proceed by a different mechanism. It also points out dramatically the pitfalls one may encounter in stability testing when the Arrhenius plot is extrapolated from higher to lower temperatures. The activation energy in this study (31.8 Kcal/mole) compares favorably to that found by Webb, *et al* (1), at lower temperatures (31.0 Kcal/mole). Thus, even though the activation energies for the different temperature ranges are similar, extrapolation of the plot from higher temperatures would yield higher rate constants than the actual values.

A further check on the kinetic expressions was obtained by employing the integrated Eqs 11 and 13. Using  $k_t$  as the assumed true rate constant for glucose breakdown and the calculated values of  $k_2$ , it is possible to calculate the amounts of 5-HMF and 5-HMF breakdown products present. These values at  $110^{\circ}$  are represented by the curves in Fig 6. The points of these plots indicate the actual experimental values. Agreement between the calculated and experimental results lends support to the idea that the reaction proceeds by a stepwise mechanism represented by the simplified scheme of

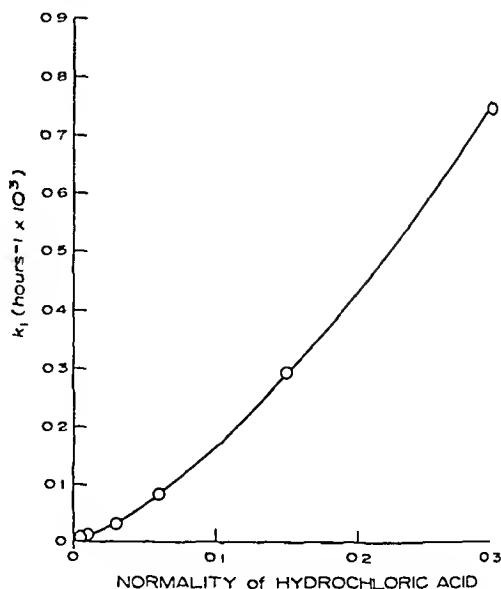


Fig 7—Variation of the rate constant ( $k_t$ ) for glucose degradation with the normality of hydrochloric acid

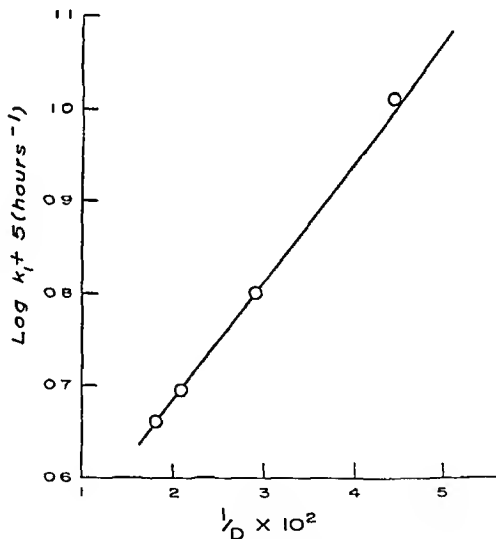


Fig 8—Variation of the logarithm of the rate constant ( $k_t$ ) for glucose degradation with the reciprocal of the dielectric constant

Eq 6. The top diagram of Fig 6 is the important one for the breakdown of glucose solutions because it shows the rate of formation of the colored compounds. Some workers have used the rate of formation of 5-HMF (center diagram of Fig 6) as a measure of the rate of discoloration, but it can be seen here that the shape of the curves are not alike. The 5-HMF formed from glucose is an intermediate in the production of colored compounds, and its rate of formation actually falls off as more colored material (humus) is formed.

**Influence of Acid on Glucose Breakdown.**—Degradation of 0.116 molar glucose solutions was carried out in varying concentrations of hydrochloric acid at a constant ionic strength and at 100°. The initial rate constant,  $k_i$ , was plotted against the normality in Fig. 7, and it is seen that the relationship is not linear. This makes the extrapolation to zero acid concentration to obtain the spontaneous rate somewhat inaccurate. However, at the very low acid concentrations the curve does assume a nearly linear form, which was also reported by Webb, *et al.* (1).

**Influence of the Dielectric Constant on the Rate Constant.**—The degradation of 0.278 molar glucose solutions at a pH of 1.27 and a temperature of 100° was carried out in dioxane-water mixtures. The dielectric constants of the solutions were calculated from data collected by Akerlof and Short (20). As a first approximation the dielectric constant of the solution was assumed to be that of the solvent. A plot of the logarithm of the initial rate constant versus the reciprocal of the dielectric constant is shown in Fig. 8. A straight line of positive slope is evidenced, a result that is predicted by theory for a reaction between a positive ion and a dipole molecule (21).

### SUMMARY

1. The degradation of glucose in acid solution was carried out at various temperatures. It was found that the rate constants calculated from the measurement of 5-HMF formation were consistently higher than those calculated from glucose depletion.

2. The rate data were fitted to a kinetic expression for first-order consecutive reactions. Good agreement between calculated and experimental values indicated that the reaction, although no doubt more complicated, can be represented satisfactorily as proceeding by this simplified scheme.

3. The rate constant at 100° was not consistent with the values found at higher temperatures. A possible change in mechanism is indicated at the lower temperatures.

4. The relationship between the rate constant and acid concentration was found not to be linear.

5. Studies in solutions of varying dielectric strength indicate that the degradation involves a reaction between a positive ion and a dipole molecule.

6. The results of this study suggest that the discoloration of glucose solutions under the conditions employed (acid solutions at elevated temperatures) follows from the breakdown of glucose and proceeds through the intermediate formation and breakdown of 5-hydroxymethylfurfural. The rate constants obtained at elevated temperatures can not be extrapolated to obtain the rate of breakdown at room temperature because of a possible difference in mechanism.

### REFERENCES

- (1) Webb, N. E., Ph.D. Thesis, Purdue University (1956); Webb, N. E., Sperandio, G. J., and Martin, A. N., *THIS JOURNAL*, **47**, 101 (1958).
- (2) Wolfson, M. L., Schuetz, R. D., and Cavaliere, L. F., *J. Am. Chem. Soc.*, **70**, 514 (1948).
- (3) Scallet, B. L., and Gardner, J. H., *ibid.*, **67**, 1934 (1945).
- (4) Joslyn, M. A., *Ind. Eng. Chem.*, **33**, 308 (1941).
- (5) Saeman, J. F., *ibid.*, **37**, 43 (1945).
- (6) Singh, B., Dean, G. R., and Cantor, S. M., *J. Am. Chem. Soc.*, **70**, 517 (1948).
- (7) Peat, S., Whelan, W. J., and Edwards, T. E., *J. Chem. Soc.*, 1955, 355.
- (8) Thompson, A., Wolfson, M. L., and Quinn, E. J., *J. Am. Chem. Soc.*, **75**, 3003 (1953).
- (9) Fetzer, W. R., Crosley, E. K., Engel, C. E., and Kirst, L. C., *Ind. Eng. Chem.*, **45**, 1075 (1953).
- (10) Bacon, E. E., and Bacon, J. S. D., *Biochem. J.*, **58**, 396 (1954).
- (11) Kent, P. W., *ibid.*, **55**, 361 (1953).
- (12) Peat, S., Whelan, W. J., Edwards, T. E., and Owens, O., *J. Chem. Soc.*, 1958, 586.
- (13) Kraus, C. A., and Vinge, R. A., *J. Am. Chem. Soc.*, **56**, 511 (1934).
- (14) Romijn, G., *Z. anorg. Chem.*, **36**, 349 (1897).
- (15) Cajori, F. A., *J. Biol. Chem.*, **54**, 616 (1922).
- (16) Laidler, K. J., "Chemical Kinetics," McGraw-Hill Book Co., New York, N. Y., 1950, p. 17.
- (17) Teunissen, H. P., *Rec. trav. Chim.*, **49**, 784 (1930).
- (18) Harcourt, A. V., and Esson, W., *Phil. Trans. Roy. Soc. London*, **156**, 193 (1866).
- (19) Esson, W., *ibid.*, **156**, 220 (1936).
- (20) Akerlof, G., and Short, O. A., *J. Am. Chem. Soc.*, **58**, 1241 (1936).
- (21) Amis, E. S., "Kinetics of Chemical Change in Solution," The Macmillan Co., New York, N. Y., 1949, p. 174.

# Theoretical Analysis of Diffusional Movement Through Heterogeneous Barriers\*

By W. I. HIGUCHI and TAKERU HIGUCHI

A number of theoretical relations dealing with diffusion through heterogeneous barriers are presented and discussed. These are expected to be useful in predicting barrier behavior in formulations such as pharmaceutical and particularly protective ointments and films. A relationship has been derived which expresses the effective permeability constant,  $P_m$ , of a two phase mixture as a function of the volume fraction and the permeability of each phase. The effect of the shape of the particles on  $P_m$  is also considered. Where data are available, agreement with theory is satisfactory. The nonstationary state behavior of a two phase heterogeneous barrier is discussed in relation to the diffusion coefficient, partition coefficient, the volume fraction, and the particle size associated with each phase. It is shown that the effectiveness of one heterogeneous barrier may be greater than another for a given time of exposure while for a longer time of exposure the reverse may be the case. The effect of a "skin" or a coat of a third phase on the internal phase particle has also been examined. Finally, the situation in which simultaneous diffusion and absorption occur is discussed and an expression for the lag time has been derived for this case.

THE PURPOSE of this study is to present some theories and relationships of pharmaceutical and pharmacological importance which deal with diffusional movement of chemical molecules through heterogeneous systems where only volume molecular diffusion is important. Although extensive experimental and theoretical studies (1) have been carried out on permeation of gases and vapors through various materials, these studies have been primarily restricted to plastic films. Diffusion in emulsions, suspensions, and powders has received relatively little attention.

Since the phenomenon of diffusion in heterogeneous materials is of great importance to workers in the fields of pharmacy and pharmacology, it was thought of interest to prepare a rather detailed theoretical analysis of this very significant mode of mass transfer. As far as we know much of what is presented here is new, representing in part an extension of a report made to the U. S. Army Chemical Corps (2).

In the majority of practical cases encountered in pharmaceutical and pharmacological areas the diffusion barrier is of heterogeneous rather than homogeneous nature. Thus the present subject is of importance in the following particular problems: (a) the design of protective ointments and films which can be employed against toxic and corrosive agents, (b) the study of drug release from pharmaceutical ointments and orally administered barrier coated dosage forms, and (c) the study of the absorption of drugs through skin and other passive biological membranes.

The aspect of this subject which was of par-

ticular interest to this laboratory was in connection with the understanding of the protective action of ointments (3). It is intended that the present report provide a clarification of the important variables rather than a rigorous mathematical treatment of this problem. The latter is impossibly difficult in many cases due to the complexity of the picture. It is assumed that the reader is familiar with the fundamentals of the diffusion problem. Material which is adequately covered by standard texts on diffusion such as those by Jost and Barrer (1), is not reviewed here but is only referred to when needed. This applies primarily to the understanding of Fick's law of diffusion and its application.

In this report the discussion will be in two parts. The first section will deal with steady state diffusion while the second part will treat the nonstationary state aspects of diffusion. As it will be seen, the effectiveness of a heterogeneous barrier predicted by a steady state analysis is not necessarily a measure of its time-dependent qualities.

## STEADY STATE DIFFUSION THROUGH EMULSION AND SUSPENSION BARRIERS

For the discussion the diagram given in Fig. 1 will be followed. On the left side of the barrier of thickness  $h$  and cross sectional area of unity is a well stirred solution of the penetrating agent at a constant concentration  $C = C_0$ , activity  $a = a_0$  (referred to the pure liquid agent as unity),<sup>1</sup> and activity coefficient  $\gamma_0$ . On the right side of the barrier is the solution of the agent  $a = a_h$ . It is assumed that  $h \ll$

\* Received December 11, 1959, from the University of Wisconsin, School of Pharmacy, Madison 6.

This work was supported by the Directorate of Medical Research, Chemical Warfare Laboratories, Army Chemical Center, Md., under Contract DA 18-108-CML-5753.

<sup>1</sup> Following the Hildebrand convention, we take activity = 1 for the pure liquid (or supercooled liquid). Then  $K_0$  is equal to the ratio of the activity coefficients. This convention is preferable to any other for our purposes.

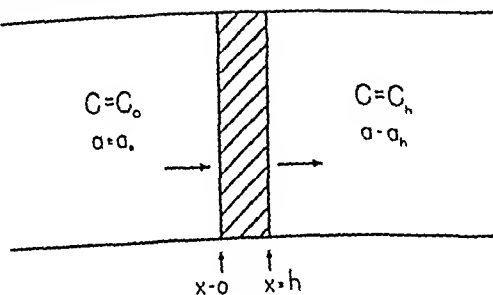


Fig 1—Diffusion through barrier

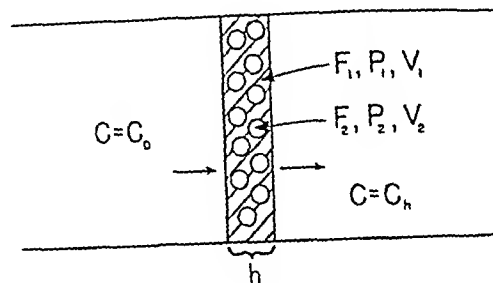


Fig 2—A two phase diffusion barrier

1 and that both Henry's law and Fick's law are obeyed throughout the barrier

If the barrier is homogeneous and if in the barrier the diffusion coefficient and the activity coefficient are  $D$  and  $\gamma$ , respectively, the rate of permeation for the case of similar solvents on both sides of the barrier is

$$p = \frac{D}{\gamma} \frac{da}{dx} = \frac{D\gamma_o(C_o - C_h)}{\gamma h} = \frac{DK_o(C_o - C_h)}{h} \quad (\text{Eq 1a})$$

where  $K_o$  is the partition coefficient<sup>1</sup> of the agent for the two media. The product  $DK_o$  may be set equal to

$$DK_o = P$$

where  $P$  is the permeability constant for the system

For the more general case of the solvents differing on both sides of the barrier, the rate of permeation would be

$$p = \frac{P_o C_o + P_h C_h}{h} \quad (\text{Eq 1b})$$

where  $P_o$  and  $P_h$  refer to the two solvents

**General Case**—The rigorous mathematics for the steady state diffusion problem for a barrier composed of one or more phases randomly or otherwise distributed in another phase is not available nor is it readily derivable. Following the analogous electrostatic problem (4-6) it is possible, however, to obtain approximate relationships between  $P_m$ , the effective permeability constant of the barrier, and permeability constants and the volume fractions of the individual phases. In what follows, expressions for  $P_m$  for random suspensions of spheres and spheroids are derived following the method of one of the authors (5) based on dielectric constant data of powders and suspensions

Figure 2 is a schematic illustration of the model to be used. For simplicity, only the case of a mixture of two isotropic phases will be treated, although the model may be easily extended to the general case of many phases. Also, it is assumed that the particles of the internal phase are uniform in shape and size, immobile in the barrier, small compared to the thickness of the barrier, but large enough to be considered phases. The barrier, then, is composed of an external phase, 1, and an internal phase, 2. The respective agent activity coefficients and diffusion coefficients are  $\gamma_1$ ,  $\gamma_2$ ,  $D_1$ , and  $D_2$  in the two phases. The average, i.e., the macroscopic, activity gradient of the agent in the barrier, the thickness of which is small compared to its volume, is of the magnitude

$$F_o = \frac{a_o - a_h}{h}$$

and is directed in the  $x$ -direction. The rate of steady state permeation is then

$$p = \frac{D_m}{h \gamma_m} (a_o - a_h) = P_m F_o$$

where  $D_m$  and  $\gamma_m$  are the effective diffusion coefficient and the effective activity coefficient of the penetrating agent with respect to the barrier. Boundary layer diffusion will be neglected and it will be assumed that the rate of interphase transport of the agent at the boundaries is rapid compared to volume diffusion.

In any macroscopic portion of the barrier one finds that the following is true

$$\frac{D_m F_o}{\gamma_m} = \frac{D_1}{\gamma_1} V_1 F_1 + \frac{D_2}{\gamma_2} V_2 F_2$$

or

$$P_m F = P_1 V_1 F_1 + P_2 V_2 F_2 \quad (\text{Eq 2})$$

where  $F_1$  and  $F_2$  are the average activity gradients of the penetrant in phase 1 and phase 2, respectively, and  $V_1$  and  $V_2$  are the respective volume fractions of the two phases. Vectorially,  $F_1$  and  $F_2$  are directed in the same direction as  $F_o$ , i.e., in the  $x$ -direction. Equation 2 states that the rate of permeation of the agent in the barrier is equal to the sum of the rates through the two phases. Also by definition, the following is true

$$F = V_1 F_1 + V_2 F_2 \quad (\text{Eq 3})$$

It is worth while to emphasize at this point that in the development of the theory it is the activity rather than the concentration of the agent which is the driving force or the potential for diffusion. It is the activity gradient rather than the concentration gradient which is the field. A solute will diffuse from a region of low concentration to a region of high concentration if the activity of the solute is less in the high concentration region.

**Internal Phase Consisting of Random Spheres.**—

If the internal phase is composed of a system of randomly distributed uniform isotropic spheres and if it is assumed that each sphere is placed in a parallel and uniform activity gradient  $F$ , then according to the solution of Laplace's equation with the proper boundary conditions (5)

$$F = \left( \frac{3P_1}{2P_1 + P_2} \right) F \quad (\text{Eq 4})$$



# Theoretical Analysis of Diffusional Movement Through Heterogeneous Barriers\*

By W. I. HIGUCHI and TAKERU HIGUCHI

A number of theoretical relations dealing with diffusion through heterogeneous barriers are presented and discussed. These are expected to be useful in predicting barrier behavior in formulations such as pharmaceutical and particularly protective ointments and films. A relationship has been derived which expresses the effective permeability constant,  $P_m$ , of a two phase mixture as a function of the volume fraction and the permeability of each phase. The effect of the shape of the particles on  $P_m$  is also considered. Where data are available, agreement with theory is satisfactory. The nonstationary state behavior of a two phase heterogeneous barrier is discussed in relation to the diffusion coefficient, partition coefficient, the volume fraction, and the particle size associated with each phase. It is shown that the effectiveness of one heterogeneous barrier may be greater than another for a given time of exposure while for a longer time of exposure the reverse may be the case. The effect of a "skin" or a coat of a third phase on the internal phase particle has also been examined. Finally, the situation in which simultaneous diffusion and absorption occur is discussed and an expression for the lag time has been derived for this case.

THE PURPOSE of this study is to present some theories and relationships of pharmaceutical and pharmacological importance which deal with diffusional movement of chemical molecules through heterogeneous systems where only volume molecular diffusion is important. Although extensive experimental and theoretical studies (1) have been carried out on permeation of gases and vapors through various materials, these studies have been primarily restricted to plastic films. Diffusion in emulsions, suspensions, and powders has received relatively little attention.

Since the phenomenon of diffusion in heterogeneous materials is of great importance to workers in the fields of pharmacy and pharmacology, it was thought of interest to prepare a rather detailed theoretical analysis of this very significant mode of mass transfer. As far as we know much of what is presented here is new, representing in part an extension of a report made to the U. S. Army Chemical Corps (2).

In the majority of practical cases encountered in pharmaceutical and pharmacological areas the diffusion barrier is of heterogeneous rather than homogeneous nature. Thus the present subject is of importance in the following particular problems: (a) the design of protective ointments and films which can be employed against toxic and corrosive agents, (b) the study of drug release from pharmaceutical ointments and orally administered barrier coated dosage forms, and (c) the study of the absorption of drugs through skin and other passive biological membranes.

The aspect of this subject which was of par-

ticular interest to this laboratory was in connection with the understanding of the protective action of ointments (3). It is intended that the present report provide a clarification of the important variables rather than a rigorous mathematical treatment of this problem. The latter is impossibly difficult in many cases due to the complexity of the picture. It is assumed that the reader is familiar with the fundamentals of the diffusion problem. Material which is adequately covered by standard texts on diffusion such as those by Jost and Barrer (1), is not reviewed here but is only referred to when needed. This applies primarily to the understanding of Fick's law of diffusion and its application.

In this report the discussion will be in two parts. The first section will deal with steady state diffusion while the second part will treat the nonstationary state aspects of diffusion. As it will be seen, the effectiveness of a heterogeneous barrier predicted by a steady state analysis is not necessarily a measure of its time-dependent qualities.

## STEADY STATE DIFFUSION THROUGH EMULSION AND SUSPENSION BARRIERS

For the discussion the diagram given in Fig. 1 will be followed. On the left side of the barrier of thickness  $h$  and cross sectional area of unity is a well stirred solution of the penetrating agent at a constant concentration  $C = C_0$ , activity  $a = a_0$  (referred to the pure liquid agent as unity),<sup>1</sup> and activity coefficient  $\gamma_0$ . On the right side of the barrier is the solution of the agent  $a = a_h$ . It is assumed that  $h \ll$

\* Received December 11, 1959, from the University of Wisconsin, School of Pharmacy, Madison 6.

This work was supported by the Directorate of Medical Research, Chemical Warfare Laboratories, Army Chemical Center, Md., under Contract DA 18-108-CML-5753.

<sup>1</sup> Following the Hildebrand convention, we take activity = 1 for the pure liquid (or supercooled liquid). Then  $K_0$  is equal to the ratio of the activity coefficients. This convention is preferable to any other for our purposes.

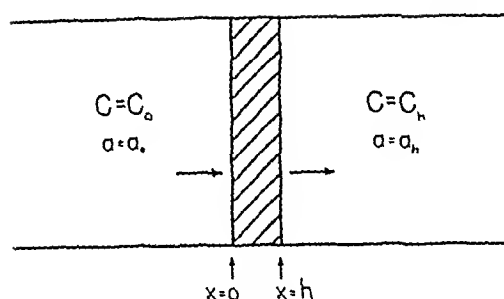


Fig. 1.—Diffusion through barrier.

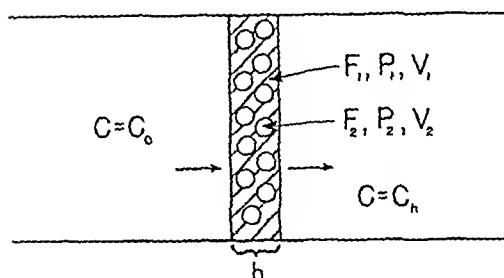


Fig. 2.—A two-phase diffusion barrier.

1 and that both Henry's law and Fick's law are obeyed throughout the barrier.

If the barrier is homogeneous and if in the barrier the diffusion coefficient and the activity coefficient are  $D$  and  $\gamma$ , respectively, the rate of permeation for the case of similar solvents on both sides of the barrier is

$$p = \frac{D}{\gamma} \frac{da}{dx} = \frac{D\gamma_o(C_o - C_h)}{\gamma h} = \frac{DK_a(C_o - C_h)}{h} \quad (\text{Eq. 1a})$$

where  $K_a$  is the partition coefficient<sup>1</sup> of the agent for the two media. The product  $DK_a$  may be set equal to

$$DK_a = P$$

where  $P$  is the permeability constant for the system

For the more general case of the solvents differing on both sides of the barrier, the rate of permeation would be

$$p = \frac{P_o C_o + P_h C_h}{h} \quad (\text{Eq. 1b})$$

where  $P_o$  and  $P_h$  refer to the two solvents

**General Case.**—The rigorous mathematics for the steady state diffusion problem for a barrier composed of one or more phases randomly or otherwise distributed in another phase is not available nor is it readily derivable. Following the analogous electrostatic problem (4-6) it is possible, however, to obtain approximate relationships between  $P_m$ , the effective permeability constant of the barrier, and permeability constants and the volume fractions of the individual phases. In what follows, expressions for  $P_m$  for random suspensions of spheres and spheroids are derived following the method of one of the authors (5) based on dielectric constant data of powders and suspensions.

Figure 2 is a schematic illustration of the model to be used. For simplicity, only the case of a mixture of two isotropic phases will be treated, although the model may be easily extended to the general case of many phases. Also, it is assumed that the particles of the internal phase are uniform in shape and size, immobile in the barrier, small compared to the thickness of the barrier, but large enough to be considered phases. The barrier, then, is composed of an external phase, 1, and an internal phase, 2. The respective agent activity coefficients and diffusion coefficients are  $\gamma_1$ ,  $\gamma_2$ ,  $D_1$ , and  $D_2$  in the two phases. The average, i. e., the macroscopic, activity gradient of the agent in the barrier, the thickness of which is small compared to its volume, is of the magnitude

$$F_o = \frac{a_o - a_h}{h}$$

and is directed in the  $x$ -direction. The rate of steady state permeation is then

$$p = \frac{D_m}{h \gamma_m} (a_o - a_h) = P_m F_o$$

where  $D_m$  and  $\gamma_m$  are the effective diffusion coefficient and the effective activity coefficient of the penetrating agent with respect to the barrier. Boundary layer diffusion will be neglected and it will be assumed that the rate of interphase transport of the agent at the boundaries is rapid compared to volume diffusion.

In any macroscopic portion of the barrier one finds that the following is true:

$$\frac{D_m F_o}{\gamma_m} = \frac{D_1}{\gamma_1} V_1 F_1 + \frac{D_2}{\gamma_2} V_2 F_2$$

or

$$P_m F_o = P_1 V_1 F_1 + P_2 V_2 F_2 \quad (\text{Eq. 2})$$

where  $F_1$  and  $F_2$  are the average activity gradients of the penetrant in phase 1 and phase 2, respectively, and  $V_1$  and  $V_2$  are the respective volume fractions of the two phases. Vectorially,  $F_1$  and  $F_2$  are directed in the same direction as  $F_o$ , viz. in the  $x$ -direction. Equation 2 states that the rate of permeation of the agent in the barrier is equal to the sum of the rates through the two phases. Also by definition, the following is true:

$$F_o = V_1 F_1 + V_2 F_2 \quad (\text{Eq. 3})$$

It is worth while to emphasize at this point that in the development of the theory it is the activity rather than the concentration of the agent which is the driving force or the potential for diffusion; it is the activity gradient rather than the concentration gradient which is the field. A solute will diffuse from a region of low concentration to a region of high concentration if the activity of the solute is less in the high concentration region.

**Internal Phase Consisting of Random Spheres.**—If the internal phase is composed of a system of randomly distributed uniform isotropic spheres and if it is assumed that each sphere is placed in a parallel and uniform activity gradient,  $F_1$ , then according to the solution of Laplace's equation with the proper boundary conditions (5)

$$F_2 = \left( \frac{3P_1}{2P_1 + P_2} \right) F_1 \quad (\text{Eq. 4})$$

However, Eq. 4 is true for a suspension only when  $P_2/P_1$  is near unity or when  $V_2$  is near zero. A more accurate approach is to assume that the particle is placed in an activity gradient

$$F_1' = F_1 + \Delta F_1 \quad (\text{Eq. 5})$$

where  $\Delta F_1$  is the contribution due to the interaction of the neighboring particles with the particle in question. The meaning of  $\Delta F_1$  may be presented in the following manner: a particle, say  $i$ , reacts to the field  $F_1' = F_1 + \Delta F_1$  and creates a perturbation field of its own. The neighboring particles react to this perturbation and create the excess field  $\Delta F_1$  at  $i$ . It was shown (5) that  $\Delta F_1$  has approximately the form

$$\Delta F_1 = \frac{\alpha^2 K F_1'}{\bar{a}^6} \quad (\text{Eq. 6})$$

for spheres where  $\bar{a}$  is the particle radius and

$$\alpha = \left( \frac{P_2 - P_1}{2P_1 + P_2} \right) \bar{a}^3$$

Then the more accurate expression for  $F_2$  is given by

$$F_2 = \left[ \frac{3P_1}{2P_1 + P_2} \right] \left[ \frac{1}{1 - K \left( \frac{P_2 - P_1}{2P_1 + P_2} \right)^2} \right] \quad (\text{Eq. 7})$$

The quantity  $K$  involves the distribution function for random spheres and is a function of  $V_2$ . It is independent of  $P_1$  or  $P_2$  if, in the derivation of Eq. 6, the terms in  $\alpha$  higher than  $\alpha^2$  could be neglected.

Then, if Eqs. 7, 3, and 2 are solved for  $P_m$

$$P_m = \frac{2P_1^2(1 - V_2) + P_1P_2(1 + 2V_2) - \frac{KP_1 \left( \frac{P_2 - P_1}{2P_1 + P_2} \right)^2 (2P_1 + P_2)(1 - V_2)}{P_1(2 + V_2) + P_2(1 - V_2) - K \left( \frac{P_2 - P_1}{2P_1 + P_2} \right)^2 (2P_1 + P_2)(1 - V_2)}}{1} \quad (\text{Eq. 8})$$

Had Eq. 4 been used instead of Eq. 7, the same expression would have resulted, but with  $K = 0$ , *viz.*, an expression analogous to the Maxwell-Rayleigh-Lorentz-Clausius-Mosotti equations (6).

Physically the difference between Eqs. 4 and 7 may be summarized as follows: Eq. 4 assumes that the induced activity gradient,  $F_2$ , of the internal phase particle in question is due wholly to the perturbation caused by the particle in question to the field,  $F_1$ . Recall, however, that  $F_1$  is the result of the presence of all the other internal phase particles as well as the presence of the external phase. On the other hand, Eq. 7 assumes that the induced activity gradient,  $F_2$ , of the particle in question must also include the effect of the modification of  $F_1$  due to the response of nearby internal phase particles to the "initial" response of the particle in question to  $F_1$ . This latter is analogous to an induced-induced effect in electrostatics.

**Internal Phase Consisting of Random Spheroids.**—In order to be able to estimate the effects of shape of the particles, the same treatment is carried out for

randomly distributed and randomly oriented spheroids.

The first step is to evaluate  $\Delta F_1$ , given by Eq. 6. As information is lacking, it will be assumed that the translational distribution function for randomly distributed ellipsoids is the same as that for a system of spheres with the same  $V_2$ .

The perturbation potential outside an ellipsoid oriented with its  $a$ -semiaxis in the direction of a uniform and parallel field  $F_1'$  is (7)

$$\phi = \frac{1}{2} \left[ \frac{\bar{a}\bar{b}\bar{c} \left( \frac{P_2 - P_1}{P_1} \right) \int_s^\infty \frac{ds}{(s + \bar{a}^2) R_s}}{1 + \frac{\bar{a}\bar{b}\bar{c}}{2} \left( \frac{P_2 - P_1}{P_1} \right) \int_0^\infty \frac{ds}{(s + \bar{a}^2) R_s}} \right] F_1' x \quad (\text{Eq. 9})$$

where  $F_1'$  is directed along the  $x$ -axis

$$R_s = [(s + \bar{a}^2)(s + \bar{b}^2)(s + \bar{c}^2)]^{1/2}$$

and  $\bar{b}$  and  $\bar{c}$  are the other two semiaxes.

If the ellipsoid is oriented with a different axis parallel to the field, an expression identical to Eq. 9 is obtained except that the appropriate semiaxis value is exchanged for  $\bar{a}$  in Eq. 9.

At distances large compared to the largest semiaxis the perturbation potential becomes (8)

$$\phi = \frac{2}{3} \left[ \frac{\bar{a}\bar{b}\bar{c} (P_2 - P_1)}{2P_1 + \bar{a}\bar{b}\bar{c}(P_2 - P_1)} \int_0^\infty \frac{ds}{(\bar{a}^2 + s) R_s} \right] \frac{F_1'}{r^3} x$$

where  $r$  is the distance to the point in question from the center of the ellipsoid.

This may be written

$$\phi = \frac{\alpha F_1'}{r^3} x$$

where

$$\alpha = \frac{1}{3} \left[ \frac{\bar{a}\bar{b}\bar{c} (P_2 - P_1)}{\frac{\bar{a}\bar{b}\bar{c}}{2} (P_2 - P_1) \int_0^\infty \frac{ds}{(\bar{a}^2 + s) R_s}} \right]$$

If the ellipsoid is randomly oriented with respect to the field  $F_1'$  with equal probability of orientations in all directions

$$\alpha = \frac{1}{9} \bar{a}\bar{b}\bar{c}(P_2 - P_1) \left[ \frac{1}{P_1 + (P_2 - P_1)A\bar{a}} + \frac{1}{P_1 + (P_2 - P_1)A\bar{b}} + \frac{1}{P_1 + (P_2 - P_1)A\bar{c}} \right] \quad (\text{Eq. 10})$$

where

$$A\bar{a} = \frac{1}{2} \bar{a}\bar{b}\bar{c} \int_0^\infty \frac{ds}{(\bar{a}^2 + s) R_s}$$

and for spheroids

$$A\bar{b} = A\bar{c} = \frac{1}{2} (1 - A\bar{a})$$

Equation 10 for  $\alpha$  is only strictly valid at large  $r$ . However it is expected to be approximately correct

for not too large  $r$ . When  $\bar{a} = \bar{b} = \bar{c}$ , Eq. 10 reduces to the  $\alpha$  expression for the sphere, viz

$$\alpha = \left[ \frac{(P_2 - P_1)}{(2P_1 + P_2)} \right] \bar{a}^3$$

The expression for  $\Delta F_1$  is then (5, 6)

$$\Delta F_1 = \frac{\frac{\alpha^2 K}{(\bar{a}\bar{b}\bar{c})^2}}{1 - \frac{\alpha^2 K}{(\bar{a}\bar{b}\bar{c})^2}} F_1 \quad (\text{Eq. 11})$$

The expression for  $F_2$  for randomly oriented ellipsoids in a uniform and parallel field  $F_1'$  is (9)

$$F_2 = \frac{1}{3} F_1' P_1 \left[ \frac{1}{P_1 + (P_2 - P_1)A_a} + \frac{1}{P_1 + (P_2 - P_1)A_b} + \frac{1}{P_1 + (P_2 - P_1)A_c} \right]$$

or

$$F_2 = \frac{1}{3} F_1 P_1 \left[ \frac{1}{1 - \frac{\alpha^2 K}{(\bar{a}\bar{b}\bar{c})^2}} \left[ \frac{1}{P_1 + (P_2 - P_1)A_a} + \frac{1}{P_1 + (P_2 - P_1)A_b} + \frac{1}{P_1 + (P_2 - P_1)A_c} \right] \right] \quad (\text{Eq. 12})$$

Now if Eqs. 2, 3, and 12 are solved for  $P_m$  with  $\bar{a} \neq \bar{b} = \bar{c}$ , i.e., for spheroids,

$$P_m = \frac{P_1 V_1 + P_2 V_2 L}{V_1 + V_2 L} \quad (\text{Eq. 13})$$

where

$$L = \frac{P_1}{3} \left[ \frac{1}{1 - \frac{\alpha^2 K}{(\bar{a}\bar{b}\bar{c})^2}} \right] \left[ \frac{1}{P_1 + (P_2 - P_1)A_a} + \frac{2}{P_1 + (P_2 - P_1)A_b = \bar{c}} \right]$$

and

$$\alpha = \frac{1}{9} \bar{a}\bar{b}\bar{c} (P_2 - P_1) \left[ \frac{1}{P_1 + (P_2 - P_1)A_a} + \frac{2}{P_1 + (P_2 - P_1)A_b = \bar{c}} \right]$$

For spheroids the expressions for  $A_a$  and  $A_b = A_c$  are simple (10)  $A_a = \frac{1}{1 - Z^2} - \frac{Z}{(1 - Z^2)^{3/2}} \cos^{-1} Z$

for  $\bar{a} < \bar{b} = \bar{c}$  and  $Z = a/\bar{b}$ ,  $A_a = -\frac{1}{Z^2 - 1} + \frac{Z}{(Z^2 - 1)^{3/2}} \ln [Z + (Z^2 - 1)^{1/2}]$  for  $\bar{a} > \bar{b} = \bar{c}$  and  $Z = \bar{a}/\bar{b}$ . In Table 1 some values for  $A_a$  and  $A_b = A_c$  are given

**Comparison of Theoretical Predictions With Experimental Data.**—Unfortunately, diffusion data over a wide range of  $P_2$  and  $P_1$  do not appear to be available in the literature. However, the analysis (5) of dielectric constant data for powders and suspensions over a wide range demonstrated that

TABLE I—CALCULATED VALUES OF  $A$  FOR SPHEROIDS

$z$	$A_a$	$A_b = A_c$
10	0.020	0.490
5	0.059	0.471
2	0.173	0.413
0.5	0.531	0.235
0.2	0.750	0.125
0.1	0.860	0.070
0.01	0.984	0.008

Eq. 8 with  $K = 0.78$  is a satisfactory representation of the effective dielectric constant of a two phase mixture in which the particle shapes of the internal phase are not too different from spherical. As the mathematics for steady state dielectric polarization and for steady state one component diffusion are identical as long as saturation effects and interfacial effects are negligible, the conclusions regarding one problem should apply to the other.

Although, according to the theory,  $K$  was expected to be a function of  $V_2$ , fortuitous circumstances (5) made necessary a choice of only a single value of  $K$  used with Eq. 8 to permit the expression to be applicable over a wide range of  $P_2$ ,  $P_1$ , and  $V_2$ . The value of 0.78 for  $K$  was expected to absorb asphericity effects arising from actual particle shape irregularities and from particle particle contacts in the system. The  $K$  for true spheres accordingly was expected to be smaller than 0.78.

Most of the available diffusion data for heterogeneous systems in the literature apply only to the extreme case of  $P_2 = 0$ , i.e., zero permeability for the internal phase. In Fig. 3 the selected data of Carman (11) for  $P_2 = 0$  are plotted. The quantity  $q$  plotted against  $V_1$ , the porosity, is defined as  $q = V_1 P_1 / P_m$ .

The data included experiments on electrical conduction as well as diffusion. Also given in Fig. 3 are curves predicted by available theoretical formulas for mixtures. Curve A is the Maxwell-Clausius-Mosotti formula for a suspension of spheres or Eq. 8 with  $K = 0$ . Curve B is Eq. 8 with  $K = 0.78$ . Curve C is Bruggeman's formula for random spheres (4), viz

$$\frac{P_2 - P_m}{P_2 - P_1} = V_1 \left( \frac{P_m}{P_1} \right)^{1/3} \quad (\text{Eq. 14})$$

which, when  $P_2 = 0$ , becomes  $P_m = P_1 V_1^{3/2}$ . Curves D and E are plots of Eq. 13 with  $K = 0.78$  for spheroids with axial ratios  $Z = 2.0$  and  $Z = 0.5$ , respectively. Equation 13 must be regarded as one which can give only a rough prediction of the effects of shape since both the Eq. 12 for  $\alpha$  and the idea that the same  $K$  for spheres may be used for spheroids are approximate. It is expected to be useful when  $Z$  is not too different from unity.

Although the scatter in the data is large, the data for spheres (circles in Fig. 3) appear to be localized in one area. A  $K$  value somewhat smaller than 0.78 used with Eq. 8 will give satisfactory agreement with the data for spheres. This is in agreement with the idea (5) that  $K = 0.78$  does absorb an asphericity factor. The experimental lower limit for  $K$  appears to be somewhat near 0.4 to 0.5 with these data. The data for systems involving random non-spherical particles always give higher  $q$  values.

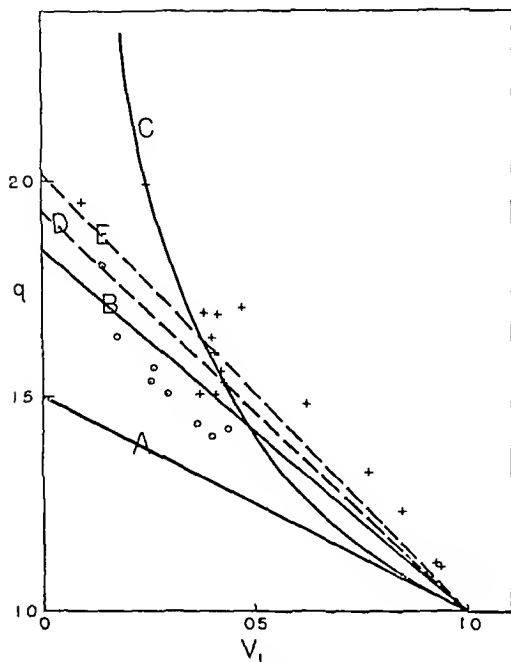


Fig 3—Plot of the tortuosity,  $q$ , against  $V_1$ . The curves  $A$ ,  $B$ ,  $C$ ,  $D$  and  $E$  refer to theory (see text) ○ Are experimental values for random spheres (uniform or a combination of large and small spheres) + Are experiments with random particles (sand, glass powder, carborundum powder, kaolin, soil, etc.)

those for spheres. This is in accord with Eq 13. Much of the data for random nonspherical particles can be explained by use of Eq 13 and a  $Z$  value not too different from unity.

Bruggeman's formula (Eq 14) for spheres predicts too large  $q$  at low  $V_1$  (or large  $V_2$ ) for these data. At large  $V_1$ , however, all formulas predict nearly the same  $q$ . It is impossible to generalize on the merits of the different formulas on the basis of these data alone. More systematic experimental work on diffusion is desirable over a range of  $P_2$  and  $P_1$  in order to establish the general applicability of any of these formulas.

It is evident that the uncertainty in calculating  $P_m$  with any formula for an arbitrary system of nonspherical particles immersed in a continuous medium is quite large. Examination of Eq 13 reveals that shape effects are not too important percentage-wise in the calculation of  $P_m$  when  $P_2/P_1$  is near unity. When, however,  $P_2/P_1 \gg 1$  or when  $P_2/P_1 \ll 1$ , shape effects appreciably influence  $P_m$ . Particularly when  $P_2/P_1 \rightarrow \infty$ ,  $P_m$  becomes very sensitive to the particle shape. Then random uniform spheroids with  $Z = 2.0$  or  $0.5$  may easily give rise to  $P_m$  values which may be twice as great as those for uniform spheres with the same  $V_2$ .

The choice of  $K = 0.78$  employed with Eq 8 was based on (5) Pearce's selected dielectric data (12) which were mostly in the range of  $0.80 > V_2 > 0.2$ . Since it is well known that Eq 8 with  $K = 0$  is a good approximation as  $V_2 \rightarrow 0$ , a  $K$  value much smaller than  $0.78$  would perhaps give better agree-

ment at  $V_2 < 0.1$ . Particularly for emulsions at low concentrations, in which the shape of the internal phase droplet is spherical and particle-particle contacts are few, a  $K$  much smaller than  $0.78$  may be more appropriate. This suggests the use of  $K = 0.78$  as an upper limit for a suspension of spheres at  $V_2 < 0.1$ . Actually at small  $V_2$  values,  $P_m$  is generally not influenced too greatly by the choice of  $K$  anyway. Therefore  $K = 0.78$  used with Eq 8 appears to provide a useful formula over all  $V_2$ .

## NONSTATIONARY STATE DIFFUSION THROUGH HETEROGENEOUS BARRIERS

**General Concepts.**—In the preceding discussions, the attention was directed only to diffusional processes occurring under quasi-equilibrium conditions such that the amount of the diffusing substance necessary to change the concentration in the barrier are of greater importance than the rate of penetration subsequent to the initial breakthrough. Thus the time it takes for water to penetrate through our shoes is of greater importance than the rate of penetration after the barriers have been wetted through. Although in most instances barriers which permit more rapid permeation under steady-state conditions also allow more rapid permeation under nonsteady-state situations, this is not always true. In this section the time lag for true diffusion in heterogeneous systems is discussed.

Taking the general problem as that illustrated in Fig 1 one may write for a one-dimensional homogeneous barrier obeying Fick's law and Henry's law

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \quad (\text{Eq 15})$$

where  $C$  is the concentration of the diffusing agent with a diffusion coefficient  $D$  in the system,  $x$  is the coordinate, and  $t$  is time. The particular problem boundary conditions are  $C = C_0$  at  $x < 0$  and all  $t$ ,  $C \sim 0$  at  $x = h$  and all  $t$ , and  $C = 0$  at  $x > 0$  and at  $t = 0$ , and it is assumed that equilibrium exists at  $x = 0$  between the barrier material and the outside phase.

The solution for this homogeneous case yields (13, 14) for the amount of material,  $M$ , penetrating the barrier per unit area per unit time

$$M = \frac{K_a D C_0 t}{h} + \frac{2h K_a C_0}{\pi^2} \sum_{n=1}^{\infty} \frac{\cos n\pi}{n^2} \left[ 1 - \exp\left(-\frac{D n^2 \pi^2 t}{h^2}\right) \right] \quad (\text{Eq 16})$$

Here  $K_a$  is the equilibrium partition coefficient of the agent in the two media defined as

$$K_a = \frac{\text{concentration of agent in barrier phase } (0 < x < h)}{\text{concentration in the outside phase } (x < 0)}$$

In Fig 4 is shown a plot of  $M/K_a C_0$  against  $Dt$  when  $h = 0.1$  cm. When  $Dt$  is sufficiently large the system has attained steady state. The intercept of the extrapolation of the steady state (linear) portion of the curve gives the well-known lag time of Barrer (14)

$$t = \lambda = h^2/6D \quad (\text{Eq 17})$$

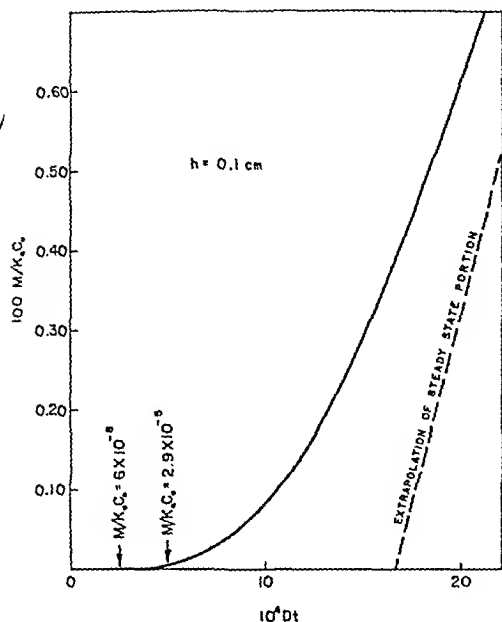


Fig. 4.—Barrier penetration with time.

which is a measure of the period required for the absorption of the agent by the barrier concomitant with the diffusion of the agent through the barrier. In protective film design this quantity is obviously important since it is roughly the measure of the time lapse necessary before the noxious agent begins to make its presence at  $x > h$ .

It would be desirable to be able to use equations similar to Eqs. 16 and 17 for heterogeneous barriers. It appears that a proper choice of an effective diffusion coefficient,  $D_m$ , and an effective partition coefficient,  $K_m$ , for the polyphase material should be sufficient to give an expression corresponding to Eq. 16.

**Emulsion and Suspension Barriers—All Phases Obeying Fick's and Henry's Laws.**—Consider the two phase problem in which the time dependent behavior of the agent in the barrier is completely defined by the diffusion coefficients, the partition coefficients, and the volume fractions corresponding to the two phases. A rigorous solution analogous to Eq. 16 appears to be impossible to obtain, but valid approximate solutions under certain conditions are possible.

At sufficiently large times, i. e., in the steady state, the effective permeability constant,  $P_m$ , may be given by an appropriate steady state mixture formula, e. g., Eqs. 8, 13, or 14; and the effective partition coefficient is given by  $K_m = K_1 V_1 + K_2 V_2$ . The effective diffusion coefficient for steady state is then

$$D_m = P_m / K_m$$

It is now important to establish the criterion for the approximate validity of these effective steady state quantities in nonstationary state problems. Essentially, the cause of the difference between the effective steady state diffusion coefficient and the effective nonstationary state diffusion coefficient is that the average paths for the diffusing molecules

may be different in the two cases. However if it can be shown that within a microscopic region of heterogeneity the nonstationary state diffusion picture is essentially that of the steady state, the effective permeability constants should be essentially the same in the two cases. This would be true if the relaxation time, i. e., the time for steady state attainment in the microscopic heterogeneous region is small compared to the time required for a unit change in concentration or activity of the agent in that region.

The smallest heterogeneous volume is that which would just include one particle of the internal phase and the corresponding volume of the external phase. If the rate determining step in the relaxation process is the diffusion into the internal phase particle, then the microscopic relaxation time is approximately (15)

$$\tau \sim \bar{a}^2 / 15 D_2 \quad (\text{Eq. 18})$$

where  $\bar{a}$  is the equivalent sphere radius of the particle. If the rate determining step is diffusion in the external phase, then the order of magnitude of  $\tau$  is

$$\tau \sim \frac{2 K_m \bar{a}^2}{K_1 D_1} \left( \frac{V_1}{V_2} + 1 \right)^{2/3} \quad (\text{Eq. 19})$$

The larger of Eqs. 18 or 19 would be a measure of the microscopic relaxation time in the heterogeneous medium. The smaller this is, the more "homogeneous" the medium appears with respect to diffusion, i. e., the more valid is the concept of the effective diffusion coefficient for use in nonstationary state problems.

The quantity  $\tau$  must now be compared with  $(\Delta t / \Delta C)_x$ , the time required for a unit change in concentration. For this purpose it is convenient to use the solution to the one dimensional barrier problem (14) when the dimension  $h$  is infinite. In the early part of the diffusion through a membrane of finite thickness, the situation is well approximated by the solution for the infinite case. Furthermore, since it is clear that for any value of  $x$ ,  $\partial C / \partial t$  will be greatest during the early stages of diffusion, it suffices to compare the relative magnitudes of  $\tau$  and  $(\Delta t / \Delta C)_x$  for the infinite barrier problem at small values of  $t$ .

The solution to the infinite barrier problem (14) is

$$C = K_m C_0 \left[ 1 - \frac{2}{\pi^{1/2}} \int_0^{\frac{\pi}{2}} (D_m t)^{1/2} \exp(-y^2) dy \right]$$

for which

$$\frac{\partial C}{\partial t} = \frac{K_m C_0 X}{2(\pi D_m)^{1/2} t^{3/2}} \exp\left(\frac{-x^2}{4 D_m t}\right)$$

The criterion for the applicability of the steady state permeability constant to nonstationary problems is then that

$$\tau \ll \frac{1}{\partial C / \partial t}$$

or that the ratio

$$R = \frac{2(\pi D_m)^{1/2} t^{3/2}}{\tau K_m C_0 x} \exp\left(\frac{x^2}{4 D_m t}\right) \gg 1 \quad (\text{Eq. 20})$$

for the range  $0 < x < h$  and  $t > 0$ .

If Eq. 20 is fulfilled for a suspension or an emulsion system, a curve identical to that in Fig. 4 should be obtained unless Henry's law or Fick's law are not

obeyed for the individual phases. If Eq 20 is not fulfilled, the  $M/K_m C_o$  values for the barrier will always be greater than those given in Fig 4, hence the barrier would be less effective as a protectant. In this case it would be extremely difficult to obtain an expression for  $M$  since the problem involves essentially an effective permeability constant which is both time and concentration dependent.

A close examination of Eqs 18, 19, and 20 will show that particle size for the internal phase usually limits the fulfillment of Eq 20 only for systems for which  $D_2 \ll D_1$  and is of practical importance when  $D_2 \ll D_1$  and  $K_2 \gtrsim K_1$ , in which case the internal phase particle may not be able to absorb rapidly enough the agent which will be diffusing predominantly in the external phase.

In Fig 5 are given plots of  $M$  against time for barriers whose internal phases are spheres or near spheres with  $h = 0.1$  cm,  $C_o = 10^{-5}$  moles  $\text{cm}^{-3} \sim 1$  atmosphere,  $K_1 = 10^{-3}$ , and  $D_1 = 10^{-6}$   $\text{cm}^2 \text{sec}^{-1}$ . In these calculations with Eq 16 it is assumed that Eq 20 is obeyed and that Eq 8 with  $K = 0.78$  is applicable. Curve A is the case where  $V_2 = 0.1$  e, the homogeneous barrier, curve B is  $V_2 = 0.3$ ,  $D_2 = 0$ , and  $K_2 = 0.1$  e, a nonabsorbing suspension, curve C is  $V_2 = 0.3$ ,  $K_2 = 10^3 K_1$ ,  $D_2 = D_1$ , 1 e, an absorbing emulsion, and curve D is  $V_2 = 0.3$ ,  $K_2 = 10^3 K_1$ , and  $D_2 = 10^{-3} D_1$ , 1 e, an absorbing solid or semisolid suspension.

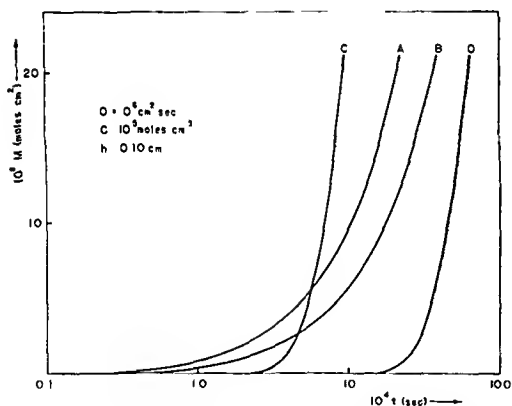


Fig 5—Penetration,  $M$ , of various heterogeneous barriers (see text) as a function of time

In terms of the effectiveness of protection case D is by far the best over all  $t$ , and case C is superior to cases A and B at low  $t$  values but is less effective for very large times. Case B is always somewhat better than case A. In terms of quantitative applicability curve C is the most approximate since, as stated in the section on "Steady State Diffusion," the uncertainty in Eq 8 is quite large when  $P_o/P_1 \rightarrow \infty$ . Examination of both Bruggeman's formula (Eq 14) and Eq 8 with  $K = 0.78$  reveals that a maximum in protection, 1 e, the maximum in time lag, for the emulsion type barrier with  $K_2 \gg K_1$  and  $P_2/P_1 \rightarrow \infty$  lies in the region of  $0.3 > V_2 > 0.2$ . Much larger or much smaller  $V_2$  values always lead to smaller lag times and therefore less protection.

It is noteworthy that for small values of  $t$ , an emulsion of the type corresponding to curve C may give

several orders of magnitude greater protection than an unfilled barrier, curve A. Therefore, for short term protection an emulsion type may most adequately serve the needs of a situation.

A particularly interesting extension of the preceding discussion results if one considers "coating" the particles or droplets of the internal phase with a third phase. Then Eq 8 may still be applicable;  $P_2$  in the formula is replaced by  $(16, 17)$

$$P_2 = \frac{[(2P_o + P_i)(\bar{a} + T)^2 - 2(P_o - P_i)\bar{a}^2]}{[(2P_o + P_i)(\bar{a} + T)^2 + (P_o - P_i)\bar{a}^2]} P_o \quad (\text{Eq 21})$$

where  $P_o = K_o D_o$  is the permeability of the outside phase (coat material),  $P_i = K_i D_i$  is the permeability of the inside material,  $\bar{a}$  = radius of the inside sphere, and  $T$  = thickness of the shell.

In real suspensions or emulsions one may essentially have a third phase "coat" or shell at the boundary of the two main phases as a result of (a) adsorption layers of foreign material, (b) reaction (oxidation) at the interface resulting in a "skin," or (c) a boundary layer with markedly different properties from either the bulk external or the bulk internal phases. In protective barrier formulation there is the possibility of actually "coating" on a third phase layer to decrease the steady state permeability while maintaining the time dependent qualities of the barrier. It can be shown from Eqs 21, 8, and 16 that a third phase coating of low permeability on emulsion droplets of high  $K_2$  may improve barrier effectiveness, for example, from that given by curve C in Fig 5 to better than that represented by curve D.

**Suspensions and Emulsions Not Obeying Fick's or Henry's Law.**—The situation when the individual phases deviate from Fick's law or Henry's law is exceedingly complicated and generally not tractable. There are, however, certain instances of great practical importance with respect to barrier design which may be handled in an approximate fashion.

Consider the system in which the internal phase is composed of particles which may adsorb molecules of the diffusing agent at its surface according to Langmuir's expression (18)

$$m = \frac{k_1 k_2 C}{1 + k_1 C} \quad (\text{Eq 22})$$

where  $m$  is the amount of adsorbate per unit mass of adsorbent,  $C$  is the equilibrium concentration of the adsorbate, and  $k_1$  and  $k_2$  are the usual constants. If the internal phase material should be characterized by a high  $k_1$ , then  $m \sim k_2$ . Assume that the rate of adsorption is rapid compared to volume diffusion, 1 e, the condition analogous to Eq 20 is fulfilled for this situation.

For such a system, the concentration gradient of the penetrating agent would approximate that shown in Fig 6. For the idealized case where essentially all of the agent entering into the system is found in the filler phase, the gradient will be strictly linear.

It is evident that the total amount of material which has entered such a barrier is the sum of the material in the external phase and that taken up by the filler. Since the average concentration of the free penetrant up to the point of maximum penetration is  $(1/2)K_1 V_1 C_o$ , where  $C_o$  is the concentration

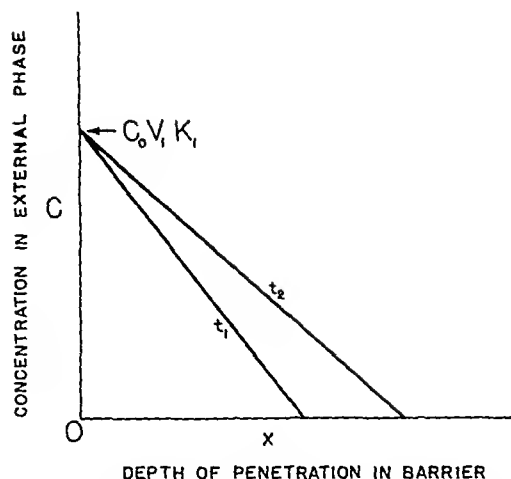


Fig. 6.—Concentration profiles in an ideal adsorption filler barrier at a time  $t_1$  and at a subsequent time  $t_2$ .

of the penetrant in the stirred solution (Fig. 1 with  $C_h = 0$ ) in equilibrium with the surface layer of the barrier. The total amount in the external phase is  $(1/2)K_1 V_1 C_0 x$ , where  $x$  is the depth of penetration.

The amount taken up by the filler up to this point is  $Ax$  where  $A$  is the amount of penetrant adsorbed by the filler per unit volume of the barrier material. The amount of penetrant,  $dW$ , necessary to effect an additional penetration,  $dx$ , would then be

$$dW = (1/2)K_1 V_1 C_0 dx + A dx$$

But,

$$\frac{dW}{dt} = -D_m \frac{dC}{dx} = D_m \frac{K_1 V_1 C_0}{x}$$

Therefore

$$\frac{D_m K_1 V_1 C_0}{x} = (1/2)K_1 V_1 C_0 \frac{dx}{dt} + A \frac{dx}{dt}$$

which in integral form is

$$V_1 K_1 D_m C_0 \int_0^\lambda dt = \int_0^\lambda x dx [(1/2)K_1 V_1 C_0 + A]$$

Therefore the lag time is

$$\lambda = \frac{h^2}{4D_m} + \frac{h^2 A}{2D_m K_1 V_1 C_0} \quad (\text{Eq. 23a})$$

or

$$\lambda = \frac{h^2 K_1 V_1}{4P_m} + \frac{h^2 A}{2P_m C_0} \quad (\text{Eq. 23b})$$

$P_m$  is given by Eq. 8 with  $P_2 = 0$  for spherical particles or by Eq. 13 for spheroidal particles. The quantity  $A$  may be replaced by  $A = C_f k_2$ , where  $C_f$  is the concentration of the filler (internal) phase and  $k_2$  is one of the constants in Langmuir's Eq. 22.

In the extreme case where none of the penetrant is adsorbed the assumed linear gradient will, of course, not hold; yet it is of some interest to note that even in this instance Eq. 23 predicts a lag time value of  $h^2/4D_m$  which is not too markedly different from the true theoretical value of  $h^2/6D_m$  from Eq. 17.

Equation 23, it is apparent, also applies to situations where the filler phase reacts chemically to immobilize or destroy the penetrating agent. This is a very common situation whose behavior the derived relationship will predict quite accurately, particularly if the reacting component is uniformly distributed either as fine particles or as solute. Indeed the same equation in a slightly different form can predict the rate of release of dispersed drug crystals, for example from ointment bases. It evidently has broad general applicability.

## GLOSSARY OF SYMBOLS

$A$	= amount of agent taken up by filler per unit volume of barrier material
$A\bar{a}, A\bar{b}, A\bar{c}$	See Eq. 10
$a$	= activity of agent
$a_o, a_h$	= activities of agent in solutions to the left of barrier and to the right of barrier, respectively
$\bar{a}$	= radius of sphere or a semiaxis of an ellipsoid
$\bar{b}, \bar{c}$	= other semiaxes of an ellipsoid
$C$	= concentration of agent
$C_o, C_h$	= concentrations of agent in solutions to the left and right of barrier, respectively
$C_f$	= concentration of filler in barrier
$D$	= diffusion constant of agent
$D_1, D_2$	= diffusion constants of agent in phases 1 and 2, respectively
$D_m$	= effective diffusion constant of agent in heterogeneous barrier
$D_i, D_o$	= diffusion constants, see Eq. 21
$F_1, F_2$	= activity gradients of agent in phases 1 and 2, respectively
$F_o$	= average activity gradient of agent in heterogeneous barrier
$F_1', \Delta F_1$	= see Eq. 6
$h$	= thickness of barrier
$K$	= $A$ coefficient in Eq. 8
$K_a$	= partition coefficient of agent, see Eq. 1a
$K_1, K_2$	= partition coefficients of agent in the solution to the left of barrier and phases 1 and 2, respectively
$K_m$	= effective partition coefficient of agent in the heterogeneous phase and the solution to the left of barrier
$K_i, K_o$	= see Eq. 21
$k_1, k_2$	= see Eq. 22
$L$	= see Eq. 13
$M$	= amount of agent having penetrated through barrier
$m$	= see Eq. 22
$n$	= an integer
$p$	= rate of permeation
$P$	= permeability constant of system for agent
$P_1, P_2$	= permeability constants for agent in phases 1 and 2, respectively (referred to solution left of barrier)
$P_o, P_h$	= permeability constants for the agent in barrier referred to the solutions to the left and the right of barrier, respectively
$P_i, P_o$	= permeability constants, see Eq. 21
$P_m$	= effective permeability constant of agent in heterogeneous barrier referred to solution to the left of barrier
$q$	= tortuosity
$r$	= radial coordinate
$s$	= a variable
$t$	= time
$T$	= thickness of coat
$V_1, V_2$	= volume fractions of phases 1 and 2, respectively
$W$	= amount of agent adsorbed by barrier
$x$	= a coordinate
$z$	= axial ratio for spheroid
$\gamma$	= activity coefficient of agent
$\gamma_1, \gamma_2$	= activity coefficients for agent in phases 1 and 2, respectively
$\gamma_o, \gamma_h$	= activity coefficients for the agent in solutions to the left and right of the barrier, respectively
$\gamma_m$	= effective activity coefficient of agent in heterogeneous barrier
$\lambda$	= lag time
$\tau$	= relaxation time
$\phi$	= perturbation potential

## REFERENCES

- (1a) Barrer, R. M., "Diffusion In and Through Solids," Cambridge University Press, London, England, 1951.
- (1b) Jost, W., "Diffusion in Solids, Liquids, and Gases," Academic Press, Inc., Publishers, New York, N. Y., 1952.
- (2) Higuchi, T., "Permeation of Mechanical Barriers by Chemical Agents," Medical Laboratories Contract Report



No. 32, Contract No. DA-18-108-CML-2576, May 1954. Chemical Corps Medical Laboratories, Army Chemical Center, Md.

(3a) Lueck, L. M., Wurster, D. E., Higuchi, T., Lemberger, A. P., and Busse, L. W., *THIS JOURNAL*, 46, 694 (1957).

(3b) Lueck, L. M., Wurster, D. E., Higuchi, T., Finger, K. F., Lemberger, A. P., and Busse, L. W., *ibid.*, 46, 698 (1957).

(3c) Finger, K. F., Lemberger, A. P., Higuchi, T., and Busse, L. W., unpublished work.

(4) Bruggeman, D. A. G., *Ann. Physik*, 24, 636 (1935)

(5) Higuchi, W. I., *J. Phys. Chem.*, 62, 646 (1958)

(6) Fricke, H., *Physiol. Revs.*, 24, 575 (1924).

(7) Stratton, J. A., "Electromagnetic Theory," McGraw-Hill Book Co., Inc., New York, N. Y., 1941, Chap. 3

(8) Lamb, H., "Hydrodynamics," Dover Publications, New York, N. Y., 1945, p. 153.

(9) Polder, D., and Van Santen, J. H., *Physica*, 12, 257 (1946).

(10) Scholte, T. G., *ibid.*, 15, 437 (1949).

(11) Carman, P. C., "Flow of Gases Through Porous Media," Academic Press, Inc., New York, N. Y., 1956, p. 48.

(12) Pearec, C. A. R., *Brit. J. Appl. Phys.*, 6, 358 (1955).

(13) Jost, W., "Diffusion in Solids, Liquids, and Gases," Academic Press, Inc., New York, N. Y., 1952, Chap. 1.

(14) Barrer, R. M., "Diffusion in and Through Solids," Cambridge University Press, London, England, 1951, Chap. 1.

(15) Gluekauf, E., *Trans. Faraday Soc.*, 51, 1540 (1955).

(16) Maxwell, E., "Electricity and Magnetism," The Clarendon Press, Oxford, England, 1892, p. 313.

(17) Fricke, H., and Curtis, H. J., *J. Phys. Chem.*, 40, 715 (1936).

(18) Langmuir, I., *J. Am. Chem. Soc.*, 38, 2221 (1916).

## Determination of Basic $\alpha$ -Epoxides\*

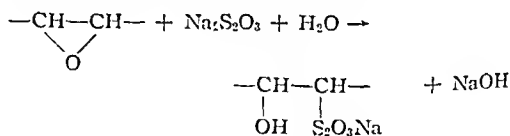
By J. B. LEARY

A common disadvantage of methods for determining basic epoxides or epoxides in the presence of a base is the necessity for determining a blank correction. A fast, direct method based on reaction with a standard solution of sodium thiosulfate is presented. The excess sodium thiosulfate is titrated with standard iodine solution. No blank correction is necessary. The stoichiometry of the reaction, the effect of temperature on the rate of reaction, precision, and other factors are described.

THE USE of certain epoxides as tumor inhibitors was first reported by Hendry, *et al.* (1, 2). The therapeutic activity of basic bis-epoxides in murine leukemia and other transplantable mouse neoplasms has been demonstrated by Johnson and Wright (3). The effects of epoxypropyl piperazine derivatives on transplanted mouse leukemia spectra have been described (4). Clinical studies of 1,4-bis(2,3-epoxypropyl)piperazine and other poly-functional alkylating agents has been reported (5). Gerzon, *et al.*, have studied the relationship between structure and antileukemic activity of some diamine bis-epoxides (6). These studies resulted in the necessity for an accurate and reproducible method for determining basic epoxides.

All of the epoxides studies were water soluble and were to be administered parenterally. This necessitated the use of a method of determination which could be used on dilute aqueous solutions. Jungnickel, *et al.* (7), have thoroughly reviewed the many methods for determining the  $\alpha$ -epoxy group and have set forth a list of recommended

methods after consideration of the physical characteristics of the epoxide to be determined and the physical form of the sample. The recommendations for dilute aqueous solutions of  $\alpha$ -epoxides include the method by Ross (8) based on esterification by thiosulfate as follows:



The method tends to be tedious since continuous titration of the liberated base with acetic acid is required to prevent hydrolysis of the thiosulfate derivative. Results obtained by this method in this laboratory on certain epoxides tended to be inaccurate (low) and were difficult to reproduce. A similar method based on the reaction between  $\alpha$ -epoxides and sodium sulfite with subsequent liberation and titration of sodium hydroxide with hydrochloric acid was reported by Kireev (9) and Swan (10) for determining various other  $\alpha$ -epoxides. A common disadvantage of the above mentioned methods is the necessity for determining a blank correction for basic epoxy compounds. Ross has mentioned that satisfactory results were obtained on more reactive compounds by carrying out the thio-

\* Received August 21, 1959, from the Analytical Development Department, Eli Lilly and Co., Indianapolis, Ind. Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

The author is grateful to Mr. E. E. Brown, Mrs. P. Cleveland, and Mr. R. E. Seroggs for many of the epoxide determinations, and to Dr. Koert Gerzon and Dr. Jack Mills for their encouragement and inspiration.

sulfate reaction in the presence of excess acetic acid. In the case of less reactive compounds, prolonged heating resulted in the decomposition of thiosulfate and deposition of sulfur. A rapid and precise method based on reaction with thiosulfate in the presence of excess acetic acid followed by titration of excess thiosulfate with iodine is herein described.

## EXPERIMENTAL

**Temperature.**—Although the Ross method required reflux temperature conditions, the rate of reaction at lower temperatures was not reported. Since lower temperatures would be more convenient, an investigation of temperature conditions was carried out. The effect of temperature on the rate of reaction of sodium thiosulfate with *N,N'*-bis(2,3-epoxypropyl)piperazine is shown in Table I.

TABLE I.—THE EFFECT OF TEMPERATURE ON THE RATE OF REACTION OF SODIUM THIOSULFATE WITH *N,N'*-BIS(2,3-EPOXYPROPYL)PIPERAZINE

Temperature, °C.	Time of Reaction	Epoxide Consumed, %
25	5 min.	55.3
	55 min.	88.8
	110 min.	93.8
	18 hr.	96.5
70	5 min.	95.7
	10 min.	98.0
	15 min.	99.4

Thus it is shown that at 70° complete reaction is obtained in fifteen minutes. All of the epoxides studied proved to react completely under these conditions.

**Stoichiometry.**—Under certain conditions the reaction between epoxides and sodium thiosulfate is quantitative, but little is known about the stoichiometry of the reaction. To answer this question an experiment was designed to determine the required excess thiosulfate to obtain quantitative results. Holding the epoxide sample size constant, various amounts of thiosulfate were added.

TABLE II.—EFFECT OF EXCESS THIOSULFATE ON EPOXIDE REACTION

Meq. $\text{Na}_2\text{S}_2\text{O}_3$ /Meq. Epoxide	Epoxide, %
1.2	93.8
1.5	95.3
1.8	96.8
2.0	97.8
2.2	98.0
2.5	98.0

The results, shown in Table II, indicate the necessity of at least a onefold excess of thiosulfate over epoxide. In other words, for every milliequivalent of epoxide in a sample, two milliequivalents of sodium thiosulfate should be added for complete reaction and quantitative results.

**Acetic Acid Excess.**—Another factor to be considered was the amount of acetic acid necessary in the reaction mixture. Theoretically equivalent amounts of acetic acid and epoxide are necessary to prevent hydrolysis of the epoxide by the sodium hydroxide produced by the primary reaction. However, the possibility remains that the reaction would not go to completion unless an excess of acetic acid was present. Holding the sample weight and sodium thiosulfate volume constant, various amounts of acetic acid were added. The results shown in Table III indicate the necessity of a 25% excess of acetic acid in the reaction mixture.

TABLE III.—EXCESS OF ACETIC ACID IN EPOXIDE REACTION

Meq. Acetic Acid/Meq. Epoxide	Epoxide, %
0.94	91.2
1.00	95.3
1.06	96.3
1.20	98.3
1.25	99.4
1.40	99.4

Table IV lists the results obtained on different basic epoxides prepared at the Lilly Research Laboratories by Gerzon, *et al.* (6).

It was noted that the purity of different lots of some of these compounds was consistently high, whereas the purity of different lots of other compounds was consistently low (see Table V). To help explain whether this phenomenon is due to a discrepancy in the method of determination or to the presence of polymeric material the conditions for optimum results were reinvestigated for compound VII. The results indicated that optimum conditions were the same for compounds I and VII.

**Reproducibility.**—Ten replicate determinations were made on a sample of compound I, and the standard deviation was found to be 1.3%.

**Reagents.**—*Iodine.*—A 0.1 *N* solution is prepared and standardized by the recommended U. S. P. XV method. *Sodium thiosulfate.*—A 0.1 *N* solution is prepared and standardized versus standard iodine solution before each series of determinations. *Starch T.S.* *Acetic acid.*—A 0.250 *N* solution is prepared by diluting 7.1 ml. of glacial acetic acid to 500 ml. with distilled water and adjusting the normality to exactly 0.250.

## METHOD

Transfer an accurately weighed sample or aliquot of an aqueous solution equivalent to approximately 1.0 meq. of epoxide to a 50-ml. Erlenmeyer flask containing 5.0 ml. of 0.25 *N* acetic acid. Add 25.0 ml. of standard 0.1 *N* sodium thiosulfate. After mixing, place the flask in a 70° temperature bath for fifteen minutes. Cool the solution quickly in an ice bath and immediately titrate with standard 0.1 *N* iodine using starch T. S. as an indicator. Calculate the amount of sodium thiosulfate consumed, and from this determine the amount of epoxide in the sample.

TABLE IV—RESULTS OBTAINED ON VARIOUS BASIC EPOXIDES

I		99.8%
II		100%
III		100%
IV		89.0%
V		99.5%
VI		96.5%
VII		91.0%
VIII		85.0%

TABLE V—RANGE OF RESULTS ON VARIOUS LOTS OF COMPOUNDS I AND VII

Compound I— Epoxide		Compound VII— Epoxide,	
Lot	%	Lot	%
1	100	1	91.2
2	100	2	91.0
3	99.9	3	89.7
4	99.8	4	89.5
5	99.4	5	89.1
6	98.0	6	89.0
7	98.0	7	88.0
8	97.8	8	87.9
9	96.5	9	87.6
10	96.4	10	87.5

## SUMMARY

A method for determination of basic epoxides has been described which is based on reaction with thiosulfate and iodimetric measurement of

the excess thiosulfate. Experimental conditions for quantitative results have been investigated. The method has been found to be accurate and reproducible.

## REFERENCES

- (1) Rose, F. L., Hendry, J. A., and Walpole, A. L., *Nature*, 165, 903(1950).
- (2) Hendry, J. A., Homer, R. J., Rose, F. L., and Walpole, A. L., *Brit J Pharmacol*, 6, 235(1951).
- (3) Johnson, I. S., and Wright, H. F., "Abstract of Papers Seventh International Cancer Congress," 1958, p. 186.
- (4) Krakoff, J. H., Burchenal, J. H., Holmberg, E., Wiegand, L., and Hemphill, S., *ibid*, p. 185.
- (5) Krakoff, J. H., Miller, D. G., Karnofsky, D. A., Burchenal, J. H., Diamond, H. D., and Craver, L. F., *ibid*, p. 294.
- (6) Gerzon, K., Cochran, J. E., Jr., White, L. A., Moahan, R., Krumkalns, E. K., Scroggs, R. E., and Mills, J., *J Med and Pharm Chem*, 1, 223(1959).
- (7) Jungnickel, J. L., Peters, E. D., Polgár, A., Weiss, F. T., "Organic Analysis," Vol. 1, Interscience Publishers Inc., New York, N. Y., 1953, pp. 127-154.
- (8) Ross, W. C. J., *J Chem Soc*, 1959, 2257.
- (9) Kireev, V. A., and Popov, A. A., *J Appl Chem U S S R*, 7, 489(1934).
- (10) Swan, J. D., *Anal Chem*, 26, 878(1954).

# The Action of Ethyl Carbamate on Oxidative Phosphorylation\*

By KWAN-HUA LEE

Ethyl carbamate inhibits  $\alpha$ -ketoglutarate oxidation and phosphorylation proportionately, but does not affect succinate oxidation or its coupled phosphorylation in rat liver mitochondrial preparations. Oxidative phosphorylation uncoupling effect is not a general mechanism of action of narcotics.

THEORIES have been proposed for the mode of action of narcotics including general anesthetics, hypnotics, and analgesics, yet none is universally accepted (1). The lack of a pronounced inhibition of oxygen consumption by the barbiturates in therapeutic concentrations suggested the possibility that these drugs may exert their effect by an uncoupling of oxidative phosphorylation. Brody and Bain (2) found that most of the few barbiturates they studied showed inhibition of both respiration and phosphate uptake in rat brain and liver mitochondrial preparations. The inhibition of phosphate uptake was greater than the inhibition of respiration and thus the P/O was decreased as drug concentrations were increased. However, their results were not in agreement with those obtained by Eiler and McEwen (3) in an earlier study on the effect of pentobarbital on oxidative phosphorylation in a rat-brain homogenate preparation. Later, Brody and Bain (4) explained that the "phosphorylation uncoupling effect" of barbiturates can only be demonstrated in isotonic sucrose mitochondrial preparations but not in water or saline homogenate preparations. Recently, Low, *et al.* (5), reported that amobarbital at a concentration of  $1.8 \times 10^{-3} M$  blocks completely those mitochondrial oxidations which proceed via pyridine nucleotides but do not affect the oxidation of succinate nor the associated phosphate uptake.

In this paper, the results of the studies on the effect of urethane (ethyl carbamate), a representative of another class of hypnotics, on oxidative phosphorylation in rat liver mitochondrial preparations are reported. We selected  $\alpha$ -ketoglutarate and succinate as two representative substrates with different oxidative pathways. It was found that when  $\alpha$ -ketoglutarate was used

as the substrate, urethane inhibited respiration and phosphorylation proportionately even at a higher concentration of drug (0.3 *M*) where respiration was reduced to only one-half of the control. When succinate was used as the substrate, the respiration and phosphate uptake were slightly inhibited at lower concentrations of urethane (0.05–0.1 *M*) and the inhibitions were not further increased as the concentration of urethane was increased. When urethane concentration was greater than 0.3 *M*, there was a sharp fall of P/O regardless of the substrate used. These results are discussed in the text.

## METHODS AND MATERIALS

**Preparation of Mitochondria.**—Ten grams of liver were quickly excised from decapitated albino rats and immediately minced in an ice-chilled glass mortar and then ground into a paste. Four volumes of 0.32 *M* sucrose solution were introduced into the paste with gentle stirring. This suspension was then transferred into a Dounce homogenizer (6), chilled in an ice bath, and homogenized with eight passes of the "loosely fitted" plunger. The homogenate was then diluted with an equal volume of a 0.25 *M* sucrose solution and then centrifuged in a Sorvall refrigerated centrifuge at  $700 \times g$  for ten minutes. The supernatant was collected and centrifuged at  $13,000 \times g$  for ten minutes. The residue was washed with 20 ml. of 0.25 *M* sucrose solution and then centrifuged at  $13,000 \times g$  for another ten minutes. The washing of the residue was repeated once more. The final residue was evenly suspended in 10 ml. of 0.25 *M* sucrose solution and used as mitochondrial preparation. During the decantation of the supernatants, attempts were made to get rid of all of the fluffy substances. All of the above procedures were carried out at ice temperature.

**Measurement of Oxidative Phosphorylation.**—Double-armed Warburg vessels in duplicates chilled in an ice bath were charged with ice cold reaction mixture and the urethane solution of various concentrations in the main compartment. In one of the side arms was added 0.3 ml. of a 70% trichloroacetic acid solution and to the other 0.5 ml. of hexokinase in glucose solution. A small roll of filter paper was placed in the center well containing 0.2 ml. of 20% KOH solution. Mitochondrial preparation was the last one added to the main compartment. After a ten-minute period of temperature equilibrium at 27°, trichloroacetic acid was tipped in the blank control flasks and hexokinase was tipped in the rest of the flasks. Oxygen uptake was measured for twenty minutes. The reaction was

\* Received December 31, 1959, from the University of California, School of Pharmacy, San Francisco.  
Read at the Fourth Pan-American Congress of Pharmacy and Biochemistry, Washington, D. C., November 3–9, 1957.

stopped by tipping in the trichloroacetic acid in the side arm. Five milliliters more of 7% trichloroacetic acid solution was added to each flask and the mixture was centrifuged. The inorganic phosphate content in the clear extract was determined according to Fiske and Subbarow (7).

Hexokinase used in this experiment was prepared from yeast and purified to the 3a stage as described by Berger, *et al.* (8). Crystalline ATP sodium salt was purchased from California Foundation for Biochemical Research. Ethyl carbamate was a Merck U. S. P. preparation which had been recrystallized three times.  $\alpha$ -Ketoglutaric acid was a product of Nutritional Biochemicals Corporation, and was recrystallized twice from ethyl propionate in order to remove the trace amount of succinic acid usually present according to Price (9). Other chemicals used were Merck C. P. grade preparations. All of the solutions were adjusted to pH 7.4 if necessary.

RESULTS AND DISCUSSION

The effect of urethane on respiration and phosphate uptake in rat liver mitochondrial preparation with  $\alpha$ -ketoglutarate or succinate as the substrate is presented in Tables I and II, respectively. The data are also plotted as per cent of control in Figs. 1 and 2. Data in Table I definitely indicate that, when  $\alpha$ -ketoglutarate was used as the substrate, urethane inhibited both the respiration and inorganic phosphate uptake proportionately and P/O values remained essentially constant throughout a wide range of drug concentrations. When the con-

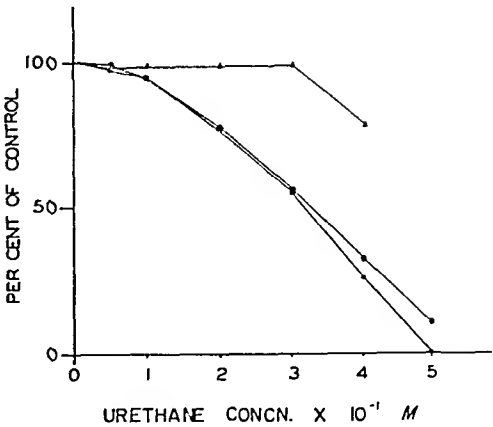


Fig. 1.—Effect of urethane on  $\alpha$ -ketoglutarate oxidation and phosphorylation.  $\blacktriangle$ — $\blacktriangle$  P/O,  $\circ$ — $\circ$  oxygen uptake,  $\bullet$ — $\bullet$  inorganic phosphate uptake.

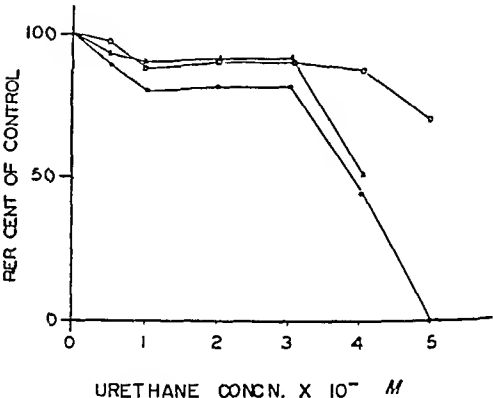


Fig. 2.—Effect of urethane on succinate oxidation and phosphorylation.  $\blacktriangle$ — $\blacktriangle$  P/O,  $\circ$ — $\circ$  oxygen uptake,  $\bullet$ — $\bullet$  inorganic phosphate uptake.

TABLE I.—EFFECT OF URETHANE ON  $\alpha$ -KETOGlutARATE OXIDATION AND PHOSPHORYLATION<sup>a</sup>

Concentration of Urethane, M	Oxygen Uptake, $\mu$ Atom	Phosphate Uptake, $\mu$ Mole	P/O
Control	7.76	28.2	3.65
0.05	7.73	27.2	3.51
0.20	6.02	21.4	3.57
0.30	4.36	15.5	3.58
0.40	2.52	7.15	2.84
0.50	0.77	0.0	...

<sup>a</sup> Each Warburg vessel contained the following substances in micromoles: ATP 6, IP 38.5, Cyt. C  $3.6 \times 10^{-2}$ ,  $MgSO_4$  23, KF 30,  $\alpha$ -ketoglutarate 30, malonate 15, urethane 0-1500, rat liver mitochondria 0.5 ml. The final volume was 3.0 ml., including 0.1 ml. hexokinase and 9 mg. glucose in 0.4 ml. 0.25 M sucrose solution.

TABLE II.—EFFECT OF URETHANE ON SUCCINATE OXIDATION AND PHOSPHORYLATION<sup>a</sup>

Concentration of Urethane, M	Oxygen Uptake, $\mu$ Atom	Phosphate Uptake, $\mu$ Mole	P/O
Control	10.3	19.3	1.87
0.05	9.92	17.2	1.73
0.1	9.0	15.0	1.67
0.2	9.25	15.6	1.70
0.3	9.20	15.6	1.70
0.4	8.90	8.55	0.96
0.5	7.12	0.0	...

<sup>a</sup> The reaction mixture was essentially the same as described in Table I except 30 micromoles of Na-succinate was used instead of  $\alpha$ -ketoglutarate and malonate.

centration of urethane reached 0.3 M, the respiration was reduced to only one-half of the control level and the P/O was still as high as the control. When succinate was used as the substrate (see Table II and Fig. 2) there was a slight decrease in P/O at lower concentrations of urethane (0.05-0.1 M), but the P/O was not further decreased as the concentrations of urethane were increased. In both cases, when the concentration of urethane was higher than 0.3 M, there was a sharp drop of P/O. At a very high concentration of urethane (0.5 M), there was no change in phosphate concentration as compared with the blank control. The effect of urethane on respiration as studied with  $\alpha$ -ketoglutarate or succinate as substrate supports the classical work of Jowett and Quastel (10) and the later work of Greig (11) that the action of narcotics on respiration is located on the electron path between mitochondria DPN linked dehydrogenases and the common point in the respiratory chain, where the electron transport from these dehydrogenases and from succinate enter the cytochrome system, commonly referred to as Slater's factor.

This was shown by the fact that urethane inhibited respiration in rat liver mitochondrial preparation when  $\alpha$ -ketoglutarate was used as the substrate but not when succinate was used.

The present results definitely deny that an "uncoupling effect" was involved in the action of urethane or as the mode of action of narcotics in general. It is not justified to consider the effect of urethane at very high concentration as drug action. It is well known that urethane is a powerful denaturing agent. The denaturation effect of urethane at high concentrations on proteins or enzymes is a general phenomenon (12).

The inhibiting effect of very high concentration of urethane on oxidative phosphorylation observed in the present study was probably due to the denaturation action of the drug on enzyme or enzymes involved in the reaction mixture.

### SUMMARY

The effects of urethane on the oxygen uptake and phosphate uptake in rat-liver mitochondrial preparation using  $\alpha$ -ketoglutarate or succinate as substrate have been studied. Urethane inhibited  $\alpha$ -ketoglutarate oxidation but not succinate

oxidation and it did not show uncoupling effect in either case. When urethane concentration was higher than 0.3 M, there was a sharp fall of oxygen uptake and P/O; this was probably due to a general denaturation of the enzyme system.

### REFERENCES

- (1) Quastel, J. H., "Proceedings of the Third International Congress of Biochemistry, Brussels, 1955," Academic Press, Inc., New York, N. Y., 1956, p. 406.
- (2) Brody, T. M., and Bain, J. A., *Proc. Soc. Exptl. Biol. Med.*, **77**, 50 (1951).
- (3) Eiler, J. J., and McEwen, W. K., *Arch. Biochem.*, **20**, 163 (1949).
- (4) Brody, T. M., and Bain, J. A., *J. Pharm. Exptl. Therap.*, **110**, 148 (1954).
- (5) Low, H., Ernster, L., and Lundberg, O., *Acta Chem. Scand.*, **9**, 199 (1955).
- (6) Dounce, A. L., Witter, R. F., Monty, K. J., Pate, S., and Cottone, M. A., *J. Biophys. Biochem. Cytol.*, **1**, 139 (1955).
- (7) Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **81**, 629 (1929).
- (8) Berger, L., Stein, M. W., Colowick, S. P., and Cori, C. F., *J. Gen. Physiol.*, **29**, 141 (1946).
- (9) Price, C. A., *Arch. Biochem. Biophys.*, **47**, 314 (1953).
- (10) Jowett, M., and Quastel, J. H., *Biochem. J.*, **31**, 565 (1937).
- (11) Greig, M. E., *J. Pharm. Exptl. Therap.*, **91**, 317 (1947).
- (12) Koeffler, H., Johnson, F. H., and Wilson, P. W., *J. Am. Chem. Soc.*, **69**, 1113 (1947).

## The Synthesis of $\beta$ -Aminoethyl Ketones as Potential Antagonists of $\beta$ -Alanine\*

By SHU-SING CHENG† and SIGURDUR JONSSON

The organocadmium reagent was adapted to the synthesis of alkyl  $\beta$ -aminoethyl ketones from  $\beta$ -alanine. Some aryl  $\beta$ -aminoethyl ketones were synthesized from  $\beta$ -alanine through the Friedel-Crafts reaction. These two series of  $\beta$ -aminoethyl ketones were proposed as potential antagonists of  $\beta$ -alanine in the metabolism of certain microorganisms. Both the alkyl and aryl  $\beta$ -aminoethyl ketones show antibacterial effect when tested against *S. aureus* and *E. coli* *in vitro*.

THIS REPORT deals with analogs of the amino acid  $\beta$ -alanine, designed as potential antagonists of possible biochemical or chemotherapeutic interest. This amino acid is significant in biological metabolism, as shown by its presence in the molecule of pantothenic acid, which is an essential component of coenzyme A (1).

\* Received August 21, 1959, from the School of Pharmacy, University of North Carolina, Chapel Hill. Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

This investigation was supported in part by a grant from the University of North Carolina. From a thesis submitted to the Graduate School of the University of North Carolina in partial fulfillment of the requirements for the degree of Master of Science.

† Recipient of the Lunsford Richardson Pharmacy Award.

In the search for metabolic antagonists of pantothenic acid, much work has been done on the synthesis of structural analogs with pantoic or  $\beta$ -alanine moiety modifications (2, 3). These structural analogs were designed and synthesized in the hope that they might interfere with the metabolism of those microorganisms which require preformed pantothenate. Analogs of  $\beta$ -alanine itself might be of interest as possible inhibitors of those microorganisms which synthesize pantothenic acid themselves from  $\beta$ -alanine and pantoic acid.  $\beta$ -Alanine has been shown to be a growth factor for various microorganisms (4-9), but not much work has been done in synthesizing compounds that might act as antagonists or displacers thereof (10).

Numerous instances of antagonism between metabolite and antimetabolite have been observed by substituting various isosteric groups for a carbonyl group of a metabolite (11). Among these is the keto fragment —COR or —COAr (12, 13). It seemed, therefore, of interest to prepare ketone analogs,  $H_2NCH_2CH_2COR$

or  $\text{H}_2\text{NCH}_2\text{CH}_2\text{COAr}$ , of  $\beta$ -alanine as possible antagonists to  $\beta$ -alanine itself. The amino group may be left intact in the hope that it can still couple with pantoic acid to give pantothenic acid analogs with a keto fragment in place of the carboxyl group. The analogs thus formed would not be able to couple with cysteine with the result that the biosynthesis of coenzyme A in the microorganism might be inhibited. A possible mode of action similar to this has been visualized by Mika and co-workers (14) while studying the growth-inhibiting effect of  $\alpha$ -alanine on *Brucella*. Alternately, the compound might react with the enzyme involved in the coupling of pantoic acid with  $\beta$ -alanine and render it inactive.

A survey of the literature for the synthesis of amino ketones showed that none of the published methods was suitable for the general synthesis of ketones of the formula  $\text{H}_2\text{NCH}_2\text{CH}_2\text{COR}$ , where R is an alkyl group. The organocadmium reagent has been widely used for the synthesis of polyfunctional keto compounds since its introduction by Gilman and Nelson in 1936 (15); however, it seems never to have been used for the preparation of amino ketones. The synthesis of the  $\beta$ -aminoethyl alkyl ketones reported here was accomplished by the reaction of  $\beta$ -phthalimidopropionyl chloride and the proper dialkylcadmium and subsequent hydrolysis of the phthaloyl group.

The  $\beta$ -aminoethyl aryl ketones,  $\text{H}_2\text{NCH}_2\text{CH}_2\text{COAr}$ , were synthesized by the Friedel-Crafts ketone synthesis from  $\beta$ -phthalimidopropionyl chloride and benzene, or substituted benzene, and subsequent hydrolysis of the phthaloyl group. These ketones were synthesized so as to test the possibility of enhanced biological activity of the aryl radical adjacent to the carbonyl group (16).

## EXPERIMENTAL

All melting points were taken with the Fisher-Johns apparatus.

$\beta$ -Phthalimidopropionic acid was prepared according to Gabriel (17). Amide, flat needles from water, m. p. 204–205°; anilide, needles from water, m. p. 189°.

$\beta$ -Phthalimidopropionyl chloride was prepared according to Gabriel (18). A better yield (93.5%) was obtained by substituting thionyl chloride for the phosphorus pentachloride.

The  $\beta$ -phthalimidoethyl alkyl ketones prepared are listed in Table I and the alkyl  $\beta$ -aminoethyl ketones hydrochlorides obtained therefrom by hydrolysis in Table II. Representative examples of the preparation follows:

*1-Phthalimido-3-heptanone*.—The cadmium alkyl was prepared in the conventional way (19, 20) from 54.66 Gm. (0.4 mole) *n*-butyl bromide through

the Grignard reagent and replacement of the ether by 80 ml. of anhydrous benzene. The flask was cooled in ice and a solution of 65.4 Gm. of  $\beta$ -phthalimidopropionyl chloride (0.27 mole) in 300 ml. of anhydrous benzene was gradually added over a period of twenty minutes, while the mixture was stirred vigorously. The mixture solidified and the ice-bath was then removed. When heat evolution subsided, the mixture was refluxed for three hours. The mixture was then cooled in an ice bath and acidified with dilute hydrochloric acid. The clear aqueous solution was separated from the benzene solution and extracted with several portions of benzene. The benzene extracts were combined, washed with ether, several portions of a 10% solution of sodium bicarbonate, and again with water, and finally dried over anhydrous sodium sulfate. The benzene was removed *in vacuo* yielding a residue of crude 1-phthalimido-3-heptanone. The crude ketone was purified by recrystallization from alcohol-water.

*1-Amino-3-heptanone (n-Butyl  $\beta$ -Aminoethyl Ketone) Hydrochloride*.—Three grams of 1-phthalimido-3-heptanone, 10 ml. concentrated HCl, and 15 ml. glacial acetic acid were refluxed for three hours, another 10 ml. concentrated HCl added, and refluxing continued for three more hours. The mixture was concentrated *in vacuo* to remove excess acid and water added until no more precipitate formed. The precipitate was filtered off, washed with water, and the washings combined with the filtrate. The filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in the minimum amount of absolute alcohol, and ether was added dropwise until further addition caused no more precipitation. The precipitate was collected by filtration, washed with ether, and dried.

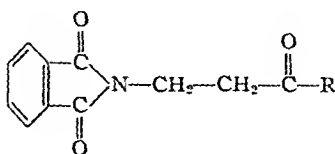
The  $\beta$ -phthalimidoethyl aryl ketones prepared are listed in Table I and the aryl  $\beta$ -aminoethyl ketone hydrochlorides, obtained therefrom by hydrolysis, in Table II. These ketones were prepared by the conventional Friedel-Crafts acyl condensation from the  $\beta$ -phthalimidopropionyl chloride, anhydrous aluminum chloride, and an excess of the aromatic hydrocarbon. The aryl  $\beta$ -aminoethyl ketone hydrochlorides were obtained by hydrolysis of the phthalimido derivative as described above.

## ANTIMICROBIAL EFFECTS

In preliminary tests all of the aminoketones here reported completely prevent the growth of *E. coli* ATCC 8739 in a synthetic medium, the 2,5-dimethylphenyl  $\beta$ -aminoethyl ketone hydrochloride and the *n*-heptyl  $\beta$ -aminoethyl ketone hydrochloride in a concentration of 250  $\gamma$ /ml.; the others at a concentration of 500  $\gamma$ /ml. The first two also completely prevent the growth of *Staph. aureus* in nutrient broth at the concentration of 100  $\gamma$ /ml. and 500  $\gamma$ /ml., respectively.

## DISCUSSION

The low yield of the alkyl  $\beta$ -phthalimidoethyl ketones can be accounted for by the volatility of the dialkylcadmium. Since the replacement of ether

TABLE I.—ALKYL AND ARYL  $\beta$ -PHthalIMIDOETHYL KETONES

R	M p, °C	Yield, %	Analyses <sup>a</sup>						Derivative M p of 2,4-Dinitro- phenyl- hydrazones, °C
			Calcd, %			Found, %			
			C	H	N	C	H	N	
<i>n</i> -Butyl	102-5	20 1 <sup>b</sup>	69.28	6.87	5.38	69.13	6.87	5.21	152-153
<i>n</i> -Hexyl	100	14 6 <sup>b</sup>	70.88	7.35	4.86	70.76	7.27	4.88	129-130
<i>n</i> -Heptyl	90	16 6 <sup>b</sup>	71.55	7.67	4.65	70.75	7.42	5.15	131-132
Phenyl	130-131 <sup>c</sup>	71	73.11	4.66	5.02	72.74	4.80	4.61	274-275
<i>p</i> -Methylphenyl	135	78	73.71	5.12	4.78	73.95	5.38	4.84	
2,5-Dimethylphenyl	136-137	71	74.26	5.54	4.56	74.23	5.76	4.50	

<sup>a</sup> Analyses by Weiler and Strauss, Oxford, England<sup>b</sup> Yield of twice recrystallized material<sup>c</sup> Literature reports 131-132°

(18), 130° (21)

TABLE II —ALKYL AND ARYL  $\beta$ -AMINOETHYL KETONE HYDROCHLORIDES  
 $H_2NCH_2CH_2COR \cdot HCl$ 

R	M p, °C	Analyses <sup>a</sup>		Derivative M p of Picrolonate °C
		Calcd, % N	Found, % N	
<i>n</i> -Butyl	142 <sup>b</sup>	8.40	8.27	179 (decompn)
<i>n</i> -Hexyl	135 <sup>c</sup>	7.23	7.2	175 (decompn)
<i>n</i> -Heptyl	132	6.74	6.77	172 (decompn)
Phenyl	126-127 <sup>d</sup>	7.54	7.57	185-200 <sup>e</sup>
<i>p</i> -Methylphenyl	193-194	7.02	6.76	186-204 <sup>e</sup>
2,5-Dimethylphenyl	173	6.56	6.76	176-187 <sup>e</sup>

<sup>a</sup> Analyses by Weiler and Strauss, Oxford, England<sup>b</sup> Literature reports 142-143° (22)<sup>c</sup> Literature reports 162° (22)<sup>d</sup> Literature reports 127° (21)<sup>e</sup> Decomposes slowly in this range without melting

with benzene is necessary for the condensation of the dialkylcadmium and the acyl chloride, the volatility of the dialkylcadmium is one of the factors limiting the yield.

The synthesis of phenyl and methylphenyl  $\beta$ -phthalimidoethyl ketones by the Friedel-Crafts acylation provides evidence that only one acyl group can be introduced into the aromatic nucleus. This coincides with the general observation that, in the Friedel-Crafts ketone synthesis, the reaction stops abruptly with the introduction of a single acyl group, so that the primary condensation product is not contaminated with products of more than one acyl group. The condensation of  $\beta$ -phthalimidopropionyl chloride with toluene gives methylphenyl  $\beta$ -phthalimidoethyl ketone. The infrared absorption spectrum of this compound shows that it is the *para*-isomer. This fact coincides with the general observation and may be properly explained as a steric effect (23).

The preliminary antibacterial tests do not show if the compounds reported here are true  $\beta$ -alanine antagonists or not. It is hoped this will be investigated at a future date. However, *E. coli*, which grows in a medium free of pantothenic acid, and hence supposedly synthesizes its own supply, is strongly inhibited by the compounds reported here. On the other hand it is not inhibited by pantoyleurine (24), a well known pantothenic acid antagonist. This lends support, indirectly, to the suggested mechanism of antibacterial effects of the  $\beta$ -aminoethyl ketones.

## REFERENCES

- (1) Lipmann, F., Kaplan, N. O., Tuttle, L. C., and Gurard, B. M., *J. Biol. Chem.*, **186**, 235 (1950).
- (2) Martin, G. J., "Biological Antagonism," The Blakiston Co., Inc., New York, N. Y., 1951, p. 225.
- (3) Fissekis, J. D., Skinner, C. G., and Shive, W., *J. Med. Pharm. Chem.*, **2**, 47 (1960).
- (4) Williams, R. J., and Rohrman, E., *J. Am. Chem. Soc.*, **58**, 695 (1936).
- (5) Mueller, J. H., and Cohen, S., *J. Bacteriol.*, **34**, 381 (1937).
- (6) Mueller, J. H., and Klotz, A. W., *J. Am. Chem. Soc.*, **60**, 3086 (1938).
- (7) Hoyer, B. H., "Some Aspects of the Physiology of Brucella Organisms in Brucellosis," American Association for the Advancement of Science, Washington, D. C., 1950.
- (8) Altenbern, R. A., and Ginoza, H. S., *J. Bacteriol.*, **68**, 570 (1954).
- (9) Wagner, R. P., and Haddox, C. H., *Am. Naturalist*, **85**, 319 (1951).
- (10) Martin, G. J., "Biological Antagonism," The Blakiston Co., Inc., New York, N. Y., 1951, p. 233.
- (11) Friedman, H. L., "First Symposium on Chemical-Biological Correlation," National Academy of Sciences, National Research Council, Publication 206, Washington, D. C., 1951, p. 296.
- (12) Ahagen, E., *Z. physiol. Chem. Hoppe-Seyler's*, **274**, 48 (1942).
- (13) Woolley, D. W., *J. Biol. Chem.*, **157**, 455 (1945).
- (14) Mika, L. A., Braun, W., Ciaccio, E., and Goodlow, R. J., *J. Bacteriol.*, **68**, 562 (1954).
- (15) Gilman, H., and Nelson, J. F., *Rec. trav. chim.*, **55**, 518 (1936).
- (16) Woolley, D. W., and Collyer, M. L., *J. Biol. Chem.*, **157**, 483 (1945).
- (17) Casati, J., *Chem. News*, **40**, 10 (1944).
- (18) Davies, R. E., and Powell, G., *J. Am. Chem. Soc.*, **67**, 1466 (1945).
- (19) Marszak, I., and Koulikas, M., *Bull. soc. chim. France*, **1956**, 93.
- (20) Berthel, F., *Ann. Chem. Phys.*, **25**, 1 (1936).
- (21) W. J. Wiley, "Antibacterial activity of metabolites," p. 77.



# A System for Measuring Contractions of the Rat Ventricle Strip\*

By DUANE G. WENZEL, IVENS A. SIEGEL, and ROBERT E. NICHOLS

A system is described for the quantitative measurement of the contractions of the electrically driven rat ventricle strip. The apparatus includes the components of the transducer-amplifier-recording system as well as a simplified muscle-holder and electrode assembly. As normally employed, the system accurately measures semi-isometric ( $>0.025$  mm. displacement) muscle contractions at a stimulus frequency of four per second. It may also be employed at other frequencies and for moderate isotonic contractions as well as for gas displacement.

THE QUANTITATIVE measurement of muscle contraction is the basis of a considerable portion of experimental pharmacology. Systems for measuring such contractions require some device by which the distance that a muscle moves or the force with which it contracts is quantitatively converted into a measurable record. Such devices are termed transducers. Systems in common use are the simple mechanical lever, the optical lever, and the various mechano-electrical systems in which the muscle displacement is quantitatively converted into an electrical signal, the signal amplified, and, in turn, used to drive an electromechanical or electro-optical recorder. For an excellent discussion of the various transducers employed in biological studies the reader is referred to the work of Machin (1).

The following paper describes a versatile system in which a variable inductance type of mechano-electrical transducer is employed. While the system to be described was designed specifically for the semi-isometric measurement ( $>0.025$  mm. displacement) of the electrically driven rat ventricular strip (2), almost any driven or spontaneously contracting muscle preparation may be studied with only minor modifications. For example, as the transducer employed uses a bellows as a spring to control the movement of the core, the apparatus may also be used to measure air or gas displacement or, by substituting a light spring for the bellows, it is effective for measuring moderate isotonic contractions.

\* Received January 30, 1960, from The University of Kansas, School of Pharmacy, Lawrence.

The investigation leading to the development of this apparatus was supported in part by a research grant (H-3842) from the National Heart Institute, Public Health Service and by a research grant from the University of Kansas.

## INSTRUMENTAL

A block diagram of the total transducer system is shown in Fig. 1. All electrical connections between the units of the system are made with shielded cable.

The transducer itself is a model 1JD2578 Westinghouse barometric rate control unit. This is rigidly mounted in and electrically insulated from a  $5 \times 6 \times 4$ -inch case. The movable transducer core is pivoted to an arm to which the muscle is attached. This arm projects through an opening cut in the front of the case. The cased transducer is in turn attached to the rack of a Babco No. 945 drill press stand. By substituting an expansion spring for the compression spring of the drill press, the vertical adjustment of the transducer case may be finely controlled with what was originally a depth-limiting screw of the drill press. This makes it possible to adjust the position of the transducer relative to the muscle chamber and to control the tension placed on the muscle.

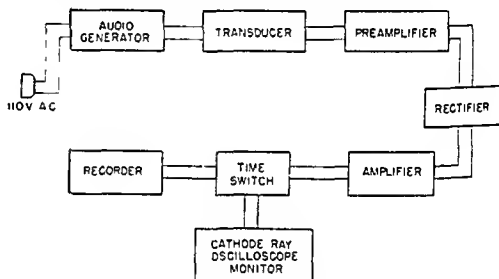


Fig. 1.—Block diagram of the electrical components of the system.

One of the disadvantages of the variable inductance transducer is a susceptibility to vibration, a drawback related to its extreme sensitivity. To minimize this effect, the mass of the apparatus is increased by substituting a solid steel rod 22 inches long by  $1\frac{1}{4}$  inches in diameter for the hollow vertical rod of the drill press, and mounting the apparatus on a base of Colorlith desk top  $20 \times 15 \times 1\frac{1}{4}$  inches. When this base is placed on several butyl rubber stoppers, the vibrational noise is minimized.

A Heathkit model AG-9A audiogenerator is used to energize the transducer coil. The rectifier operates at an audiogenerator frequency of 20 kc. and a voltage of 0.5 was found to be satisfactory. The signal from the transducer is first amplified by a Tektronix type 122 low level preamplifier with a voltage gain of 100 and a frequency response between 80 cycles and 40 kilocycles. The output of the preamplifier is then rectified in order to remove the 20 kc. carrier wave. A circuit diagram for the rectifier is shown in Fig. 2. The frequency response of this system is essentially flat between one and twenty c.p.s. The d. c. output of the rectifier is

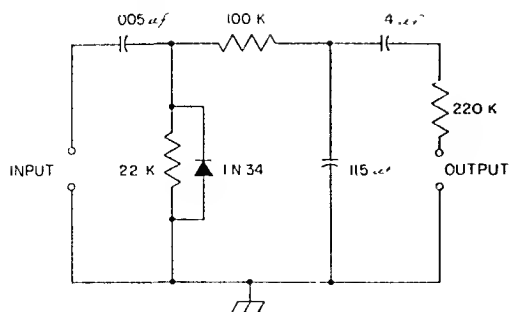


Fig. 2.—Circuit diagram of the rectifier.

further amplified by either an Epsco model 8108-B or a model 8122-A amplifier. In the experiment for which the apparatus was designed, that is to follow a stimulus frequency of four per second, either amplifier is adequate. However, for possible applications in which a relatively steady state is to be measured, the 8122-A amplifier will not provide adequate gain as the d. c. gain of this instrument is only 150. A continuously variable gain control on amplifier controls the final voltage output to an appropriate recording device. For the application to be described, it is desired to automatically record the results for a ten-second period every five minutes. This is accomplished by the use of a recycling cam timer. This timer is used to alternate the signal between a Brush model BL201 oscillograph for ten seconds and a Tektronix type 360 oscilloscope, which was used to monitor the results, for the remainder of the five minutes. For recording at frequencies of less than two per second, a Texas Instruments Recti-Riter provides a rectilinear record of greater amplitude. For studies in which frequencies higher than about 10 per second are to be employed, it is necessary to modify the system of rectification.

The linearity of the system was measured by adding weights to the transducer arm and recording the response on the oscillograph. Response was linear as shown in Fig. 3 within the approximate limits of the force exerted by the ventricular strip. The limits of linearity were not studied and should be determined if the system is to be employed for the measurement of isotonic contractions.

The remainder of the apparatus differs from similar devices previously reported in several respects and is reported in brief. The muscle chamber consists of a cylindrical vessel 10 cm. in height and 4.5 cm. in diameter. As shown in Fig. 4, a polystyrene mercury-filled electrode with a platinum contact enters the chamber at the side arm through an ampul stopper. An air stone of the type used in aquariums is similarly introduced through an opening at the bottom of the bath. The muscle holder, as seen in Fig. 4 A, is likewise made of polystyrene plastic. In order to fix the muscle mechanically and electrically to the holder, the strip is first tied at both ends with silk thread. Nylon thread should not be used as it stretches. One end of the muscle is then pulled through a small, carefully bored hole approximately 0.5 mm. in diameter in a soft rubber-ampul cap. By stretching the hole with a forceps to enlarge the opening, approximately 1 to 2 mm. of the muscle is pulled into the inside of

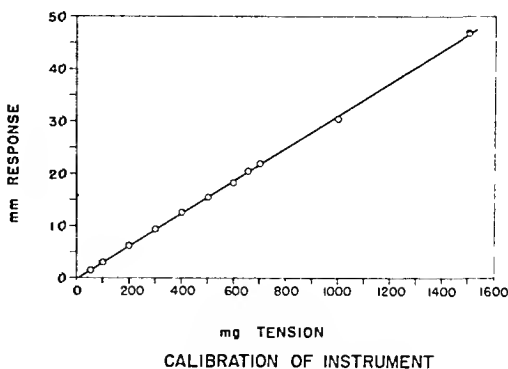


Fig. 3.—The relation between the amount of tension placed on the transducer and the response of the recorder.

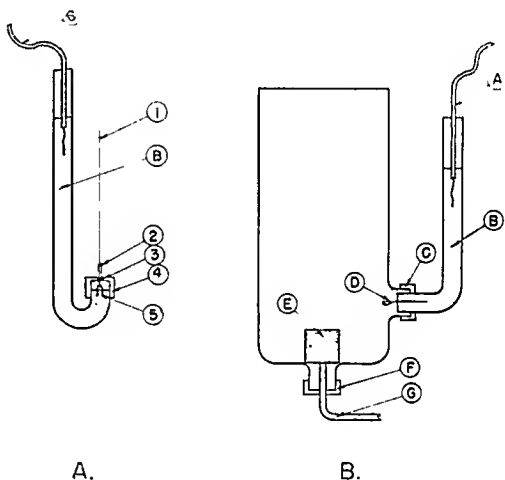


Fig. 4.—A, Muscle chamber. 1, Connection to lever arm; 2, muscle strip; 3, 0.5-mm. hole for insertion of strip; 4, rubber cap; 5, platinum cap; 6, connection to stimulator.

B, Electrode assembly. A, Connection to stimulator; B, mercury filled electrode; C, rubber cap; D, platinum electrode; E, air stone; F, rubber cap; G, oxygen supply.

the cap. The cap holding the muscle with approximately 1 cm. outside and 1 mm. inside the cap is then pushed down over the muscle holder in order that the muscle comes in contact with the electrode. The end of the muscle holder through which the electrode protrudes is expanded to such a size that the ampul cap fits very tightly. It is necessary to select an ampul cap having a sufficiently heavy top to avoid movement of the cap with each muscle contraction.

The muscle is stimulated four times per second with the output of a Tektronix type 162 waveform generator and a Type 161 pulse generator powered by a type 160A power supply. Such a system provides all the advantages of more expensive commercial biological stimulators and offers considerably more flexibility.

Temperature was controlled at  $27^\circ \pm 0.25^\circ$  by

the use of a Thermistemp temperature controller model 71 and a 2084 liquid immersion probe. Heating or cooling as required was accomplished by pumping water at a temperature differential of about 5° through 1/8-inch diameter polycetylene tubing wrapped around the muscle chamber.

## REFERENCES

- (1) Machin, K. E. "Electronic Apparatus for Biologic Research." Academic Press, New York, N. Y., 1958, p. 471
- (2) Feigen, G. A., Masuoka, D. T., Thienes, C. H., Sanders, P. R., and Sutherland, G. B., *Stanford Med. Bull.*, 17(1952)

# Studies on Bacterial Pyrogenicity II\*

## A Bacteriological Test for Pyrogens in Parenteral Solutions

By STANLEY MARCUS, CARL ANSELMO, and JOANNA LUKE

The basis and procedure for a culture method employing membrane filters to test freshly prepared parenteral solutions for pyrogenic capacity is presented. Under the limiting conditions described, solutions which yielded counts of less than 10,000 per L. were found to be nonpyrogenic by rabbit test. On the basis of laboratory and field trial, it is concluded that the bacteriological assay for the purpose described is as specific and sensitive as the rabbit assay for pyrogenicity.

WORK REPORTED (1, 2) has given support for the concept that official pyrogen tests, including the U. S. P. test, have a quantitative basis in terms of the numbers of bacteria required to induce a febrile response in rabbits. That is, a definite number of organisms must contaminate a solution before it becomes pyrogenic.

In this report a culture technique is described which has been successfully employed as an alternative for the official rabbit test for pyrogenicity of freshly prepared parenteral solutions. Results obtained are given and some implications concerning the method are explored.

## MATERIALS AND METHODS

Pyrogen tests in rabbits were carried out with sterile (autoclaved) solutions according to the techniques described in the "U. S. Pharmacopeia XV," with minor exceptions previously noted (2). Interpretations of results were in accordance with U. S. P. XV. Samples of solutions to be tested by the culture procedure were taken before

sterilization and filtered through so-called "membrane" filters (Millipore, type HA, white, grid, 47 mm.). That is, the batch of solution was prepared and either an aliquot was removed from the one sample or one or more containers of the bottled lot was sampled immediately prior to placing the remainder into the autoclave for sterilization. At least 100 ml. of each solution was filtered. Each filter was then aseptically transferred to a human (2-5%) blood agar plate and incubated at 37° to yield estimates of the numbers of living organisms present in the samples prior to sterilization. Colony counts were made after forty-eight hours incubation. Reasons for the use of the blood agar medium, incubation temperature, and time are considered in the discussion section.

Theoretical considerations as well as practical findings have led us to conclude that a freshly prepared solution yielding a count of 10,000 or less per L., under the defined conditions of the test, is nonpyrogenic as determined by rabbit assay.

## RESULTS

Freshly distilled water, collected under ordinary conditions of cleanliness in the experimental laboratory from a glass still that condensed approximately 2 L. per hour, with no unusual precautions taken to avoid air-borne contamination, was usually sterile. Such water transferred to previously washed (detergent and hot tap water) and then steamed (live-steam hose) 5-gallon glass carboy containers maintained water in a nonpyrogenic state for the duration of the test period, that is, three months. The mouths of the carboys were covered by an initially sterile 1-L. Griffin beaker. After twelve to fourteen weeks three 5-gallon specimens prepared in this fashion yielded counts of 98, 174, and 35 organisms/L. The water in each carboy was converted to 0.9% NaCl by addition of the proper amount of a U. S. P. grade reagent. To simulate hospital laboratory conditions as well as mix the solution, the water was poured from the carboy into a

\* Received September 18, 1959, from the College of Medicine, University of Utah, and Latter Day Saints Hospital, Salt Lake City.

Taken in part from a thesis submitted by C. Anselmo to the Graduate School of the University of Utah in partial fulfillment of the Master of Science degree.

This work has been aided by the American Sterilizer Foundation, Erie, Pa., and the Latter Day Saints Hospital Foundation for Medical Research.

10-gallon aluminum vat previously cleaned and then rinsed with freshly distilled water. Average plate counts after forty-eight-hours incubation on two 100-ml. samples of the finished products were 34, 62, and 49 organisms, corresponding to values of 340, 620, and 490 organisms/L. Immediately after samples were taken for bacterial counts, liter amounts were sterilized by autoclaving. Pyrogen tests in rabbits with the autoclaved samples were negative.

Freshly distilled water, collected under usual conditions of cleanliness in the central supply area of a large general hospital, was found to be sterile by membrane filter technique. This water, prepared from a still operating by connection to a steam line, was collected into a 12-gallon glass carboy. A plastic hose through which water was taken from the carboy was found to be consistently contaminated with organisms of the family *Achromobacteriaceae*. These organisms, which are not significant pyrogen producers (2), were reduced to low numbers by flushing about 2 L. of freshly distilled water through the plastic drain after washing the terminal portion of the drain with detergent in hot tap water. The tap water was not softened by ion exchange resin treatment. Freshly distilled water from this carboy was used to prepare small (4-L.) batches of 0.9% NaCl (4 batches), 5% dextrose (4 batches), or a mixture of these substances (2 batches) in a 6-L. flask; all these preparations but one contained less than 100 viable organisms/L. and all were negative in the rabbit pyrogen assay. The chemicals used in these preparations were U. S. P. grade.

Water from a Barnstead electrically powered, all metal still, rated at one gallon per hour, was collected in 5-gallon glass carboys and found to be sterile on repeated tests by membrane filter analysis. The occasional presence of one or two colonies in cultures of membrane filters through which liter amounts had been sampled was interpreted to represent transfer contamination. This water was used to rinse out the 10-gallon aluminum vat previously referred to. Following rinsing, 4 gallons of water was added to the vat and converted to 0.9% saline solution by the addition of 36.2 Gm. of U. S. P. NaCl. From the hose outlet at the bottom of the vat, successive 500-ml. samples yielded colony counts of 3, 4, and 2 organisms corresponding to estimates of 6, 8, and 4 organisms per L. The autoclaved specimens were negative for pyrogens by rabbit test. These data are summarized in Table I.

A critical evaluation of the bacteriological test for pyrogens in freshly prepared solutions was carried out in cooperation with a reputable company which prepares and sells parenteral solutions.<sup>1</sup> Samples at the plant were collected at or from: (a) the distilled water delivery outlet of the main still, (b) a collecting tank containing approximately 50 gallons of freshly distilled water, (c) this latter source after mixing in the U. S. P. grade chemicals, (d) the washed, filled, and capped 1-L. bottles ready to be autoclaved. It was predicted that the successive samples would yield cumulative colony counts. In general, this was found to be true.

<sup>1</sup> We are grateful for the cooperation of the Allied Pharmaceutical Co., Centerville, Utah.

TABLE I.—RELATION BETWEEN BACTERIAL COUNT AND PYROGEN TEST RESULTS IN VARIOUS PARENTERAL SOLUTIONS

Sample	Membrane Filter Count Colonies/L.	U. S. P. Rabbit Pyrogen Test
Glass distilled water		
Fresh	0	..
No. 1 5 gals. After 14 wks.	<100	..
No. 2 5 gals. After 13 wks.	<200	..
No. 3 5 gals. After 12 wks.	<100	..
No. 1 Converted to saline	<400	neg.
No. 1 Converted to saline	<400	neg.
No. 2 Converted to saline	<700	neg.
No. 3 Converted to saline	<500	neg.
Steam line distilled water		
Fresh (4-L. Batches)	0	..
No. 4 0.9% Saline	<100	neg.
No. 5 0.9% Saline	< 10	neg.
No. 6 0.9% Saline	< 10	neg.
No. 7 0.9% Saline	< 10	neg.
No. 8 5% Dextrose	<100	neg.
No. 9 5% Dextrose	<100	neg.
No. 10 5% Dextrose	<100	neg.
No. 11 5% Dextrose	<100	neg.
No. 12 5% Dextrose in saline	<200	neg.
No. 13 5% Dextrose in saline	<100	neg.
Barnstead distilled water		
Fresh (4-gal. Batch)	0	..
No. 14 0.9% Saline	< 10	..
	< 10	neg.
	< 10	..

For example, in one experiment, the fresh distillate was sterile, the collected distillate contained an estimated 50–100 organisms/L., the 5% dextrose solution, following mixing, yielded an estimate of 440 organisms/L., and two 1-L. bottles selected at random from the 180-L. bottle batch yielded estimated counts of 560 and 680 organisms/L. Samples of autoclaved specimens were sterile and pyrogen tests were negative. Inconsistencies in counts in 12 experiments were ascribed to logistic problems, that is, collecting and bringing the samples to the laboratory to be tested. In any event, in these experiments, as well as another 62 experiments in which cultures were carried out on samples from bottles taken from racks just prior to placing in the autoclave, counts of 240 to 3,400 organisms/L. were obtained. The distribution of these counts, in estimated number of organisms per L., is shown in Table II. The specimens included examples of the usual commercially available parenteral solutions of the saline, dextrose, and Ringer's types. All batches yielded negative pyrogen tests in rabbits.

## DISCUSSION

Since bacteriological culture tests have been indicted as unreliable for general pyrogen assay in the past (3–7) it is necessary to consider in detail the hypotheses and arguments on which are based this claim for the reliability of a culture method.

If it is recalled that all assay tests are standardized with both explicit (defined) and implicit

TABLE II.—DISTRIBUTION OF COLONY COUNTS ON 74 FRESHLY PREPARED SOLUTIONS PRIOR TO STERILIZATION

Colonies Counted <sup>a</sup> per 100-ml. Sample	Organisms Estimated, per L.	No of Specimens
24-50	240-500	2
> 50-<100	> 500-<1000	21
>100-<150	>1000-<1500	27
>150-<200	>1500-<2000	14
>200-<250	>2000-<2500	6
>250-<300	>2500-<3000	1
>300-<350	>3000-<3500	3

<sup>a</sup> Blood agar, 48 hours at 37°.

("common-sense") limits of error, then the defense of the proposed assay for pyrogens in freshly prepared parenteral solutions is more readily rationalized.

By "parenteral solution" we refer to any of the usually available salt and simple sugar solutions put up in volumes of 1 L. to as little as 5 ml. and designed for intravenous or other form of injection or infusion into man. For the present we specifically exclude consideration of vitamin, antibiotic, and other biological preparations which are administered by injection.

The object of the assay method proposed is to determine with confidence if a freshly prepared solution contains less than 10 organisms per ml. (10,000/L.) by the technique described. Under these circumstances, thermophilic, psychophilic, photosynthetic, and anaerobic bacteria will not be detected. Fastidious pathogens such as *N. meningitidis*, *H. pertussis*, or *M. tuberculosis* will not grow out. Of course, nonviable bacteria as well as other forms of microbic life (viruses and protozoa) will also go undetected. The hypothesis explored therefore is that salt and sugar solutions prepared for parenteral use, by prescribed standard methods, with freshly distilled water, will be nonpyrogenic if such solutions contain fewer than 10,000 bacteria, yeast, or mold spores per L., without respect to species of such microorganisms, when tested immediately after preparation by the method described.

Reasons for the explicit limitations to the method are derived mainly from material previously reported (1, 2). It has been found that at least 1,000 per ml. of the most pyrogenic bacteria must contaminate a solution, that is then sterilized by autoclaving, before it will yield a pyrogenic response by rabbit test. Since the potent pyrogen producers are apparently rare as compared to the usual contaminants of freshly prepared parenteral solutions, the hypothesis may be suggested that defining a count of 10 organisms per ml. ( $10^4$ /L.), rather than 1,000 per ml. ( $10^6$ /L.), as potentially pyrogenic results in a "safety factor" of about 100 times to account for nonviable pyrogenic sources in the material tested. In other words, a solution yielding a count of  $10^4$  to  $10^6$  added organisms per L. will be negative in the rabbit test. This will be true if the organisms are added, but not necessarily so if the organisms grow in the solution. This is why the test must be restricted to freshly prepared solutions.

We delineate a "freshly prepared solution," at present, as one prepared with water distilled,

used, and sterilized within a twenty-four-hour working period. The twenty-four-hour working period has been chosen arbitrarily both on the basis of trial and from theoretical considerations. Water collected overnight in previously cleansed tanks and then used for washing and solution preparation on the following day has been found to be indistinguishable by rabbit test from water collected and used during an eight-hour period. This is not surprising when we view the fact in the light of the hypothesis that difficulty with pyrogens arises from organisms added to the solution during the manufacturing process and prior to sterilization. Conclusions of this nature were reached by Seibert (8) who stated: "... there seems to be no doubt... that the pyrogen is a bacterial contaminant, as suspected by many former investigators... It is possible to free distilled water of this contamination by a proper distillation and only such fresh distillates should ever be used in the preparation of solutions for parenteral injections. If these fresh distillates are sterilized immediately and preserved sterile they will remain free from pyrogen. On the other hand, if they are once allowed to become contaminated, it is not possible to free them of pyrogen by autoclaving..." In concurring with this conclusion we amend the statement by defining "fresh distillates" as we have done and by introducing the qualitative and quantitative concepts of pyrogenicity for freshly prepared solutions. In our experience, the most frequent contaminants of distilled water (*Achromobacteriaceae*) have a low pyrogenic potential (2) and prolonged generation time under natural conditions. Also, in this regard, the dangerous heterotrophic species of *Enterobacteriaceae* do not find freshly distilled water, containing trace amounts of atmospheric gases in solutions, a choice medium should they be inoculated into the distilled water during the distillation period; and there is not sufficient time for significant numbers of organisms to accumulate in the prepared solution before it is sterilized if the working period is restricted to twenty-four hours. Of course, stills, receptacles, containers, and delivery lines must be of types which are easily and thoroughly cleaned so that no debris may be trapped anywhere to act as a culture inoculum or medium.

The hypothesis that each colony that grows out arises from a single bacterial cell is drawn from experimental observation of the satisfactory relationship between organisms added to solutions and membrane filter counts made from aliquots of these solutions.

The assumption that all microorganisms are equally pyrogenic is entirely unjustified, does not fit the available data, and is employed on the basis of being a further safety factor in the allowable count; that is, organisms that we have isolated from freshly prepared parenteral solutions, immediately prior to autoclaving, are much lower in pyrogenic potential than is indicated by the assay limits of acceptability.

It should be apparent that the proposed method is unsuitable for use with ion exchange water. Until such time that suitable methods are found for readily cleaning and sterilizing ion exchange resin columns the use of such water for parenteral preparations should be discouraged rather than guarded. At this time, the use of ion exchange water for

parenteral preparations is forbidden in U. S. P. procedure and lack of available information on the subject makes use of such water hazardous from all points of view.

The bacteriological assay procedure proposed should monitor the cleanliness of the plant operation. That is, if a normal range of bacterial counts is once established, a significantly increased count deviation from this mean should indicate the existence of an actual or potential trouble source.

Disadvantages of the procedure are apparent. A bacteriology laboratory or semblance thereof must be available. The test is a secondary type procedure based on the primary bioassay with rabbits, which itself is fallible. The time limitations of the test have been stressed, that is, unlike the rabbit test, the culture assay cannot be delayed but must be performed as soon as the solution is prepared and is ready for autoclaving. Furthermore, the results cannot be determined for forty-eight hours.

## SUMMARY

A simple culture technique, employing membrane filters (Millipore, HA) to test freshly prepared parenteral solutions for pyrogenic capacity, has been described. The basis and limiting conditions for the method are noted and discussed, as are certain advantages and disadvantages.

## REFERENCES

- (1) Marcus, S., Anselmo, C., and Perkins, J. J., *Bacteriol. Proc.*, 1958, 90.
- (2) Marcus, S., Anselmo, C., and Perkins, J. J., *Proc. Soc. Exptl. Biol. Med.*, 99, 359(1958).
- (3) Hart, E. C., and Penfold, W. J., *Brit. Med. J.*, 2, 1589 (1911).
- (4) Seibert, R. R., *Am. J. Physiol.*, 67, 90(1923).
- (5) Rademaker, L., *Ann. Surg.*, 92, 195(1930).
- (6) Probey, T. F., and Pittman, M., *J. Bacteriol.*, 50, 397 (1945).
- (7) Berger, A., Elenbogen, G. D., and Ginger, L. G., *Advances in Chem. Ser.*, 16, 168(1956).
- (8) Seibert, F. R., *Am. J. Physiol.*, 71, 621(1925).

# A Comparative Study of Theophylline Preparations: Digestive Absorption\*

By SAMUEL H. WAXLER and HERBERT B. MOY

Six theophylline preparations have been tested for their absorption from the gastrointestinal tracts of animals as reflected by blood theophylline levels. These values were obtained first from the stomach, and then from the remainder of the gastrointestinal tract. The results showed satisfactory absorption with all medications, and the wide variation inherent in oral preparations. No real superiority was evident in the six preparations when the available theophylline was the same in each drug.

IN 1949 Schack and Waxler (1) described an ultraviolet spectrophotometric method for the determination of theophylline in blood and tissue. Subsequently this method was applied to a study on groups of patients and blood theophylline levels were determined following the administration of aminophylline by intravenous, intramuscular, oral, rectal, and inhalation routes (3, 4, 5). Attention was paid primarily to the rate of appearance, the level attained, and the disappearance of the drug from the blood stream. Since that time, several papers on new oral theophylline preparations have appeared. Two points are generally stressed with these preparations: the superior blood levels, and the fewer gastrointestinal disturbances. The problem of absorption of the drug from the gastrointestinal tract has been considered only in very general terms. Since little is known, no references are

made to the differential absorption of the drug from any particular part of the tract, i. e., the stomach or the small intestine. This present study is an attempt to determine in animals the amount of absorption of theophylline from the isolated stomach in a set interval of time, and also the absorption which occurs from the intestinal tract after the theophylline material leaves the stomach.

## MATERIALS AND METHODS

In these experiments six theophylline preparations: theophylline (Searle); aminophylline (Searle); calcium theophyllinate (Searle) (2); aminophylline-aluminum hydroxide-ethyl aminobenzoate (Irwin, Neisler); aminophylline-aluminum hydroxide gel (Massengill); and choline theophyllinate (Barnes Hind); were examined for their absorption, first from the stomach of rabbits and dogs, and, subsequently, from the remainder of the intestinal tract of these animals. All of the preparations

\* Received October 27, 1959, from Stanford University, School of Medicine, San Francisco, Calif.

were given so that an equal amount of available theophylline was used. The total theophylline in each compound was computed, and this was given in the amount of 15 mg of theophylline per Kg of body weight to the rabbits, and 25 mg of theophylline per Kg to the dogs.

Forty-one rabbits weighing between 2 and 3.4 Kg were taken off feed twelve hours before the experiments. Under tribromethanol and light ether anesthesia, a small abdominal incision was made, the stomach exposed, and a permanent ligature placed at the distal end of the stomach. The prescribed amount of preparation was administered and allowed to enter the stomach.

Thirty-eight rabbits in the same weight range were prepared in a similar manner under tribromethanol and light ether anesthesia. A soft rubber-shod clamp was temporarily placed at the distal end of the stomach and the animal was given, by stomach tube, one of each of the six above-mentioned theophylline preparations. At the end of one-half hour the clamp was removed and the material allowed to continue into the small intestine.

A similar experiment was performed with dogs. The animals were anesthetized with chloralose, 100 mg per Kg of body weight subcutaneously, and kept under light ether anesthesia as required. The abdominal cavity was opened, a clamp was placed at the distal end of the stomach, and the various theophylline preparations, 25 mg per Kg, were

administered by stomach tube. The clamp was removed one hour after the administration of the drug.

A preliminary blood sample for "blank" determination was drawn prior to the administration of the drug; then serial samples were drawn through the next five to seven hours. The blood sample were analyzed for theophylline content by the method of Schack and Waxler. A Beckman DU ultraviolet spectrophotometer was used for determination of the values.

## RESULTS

The data obtained following oral administration of theophylline preparations to rabbits with a ligature at the distal end of the stomach are shown in Table I. The significant degree of absorption from the stomach after thirty minutes is reflected in the elevated blood levels. These levels were maintained and increased over the next seven hours. The range within each group is quite marked; and these variations are noted with all drugs tested.

The second phase of this experiment gives the results following temporary occlusion of the distal end of the stomach of the rabbit with a rubber-shod clamp which is removed thirty minutes after oral administration of the drug. The values at the thirty-minute mark (Table II) indicate theophylline levels obtained from absorption from the stomach

TABLE I—BLOOD THEOPHYLLINE LEVELS OBTAINED FROM ABSORPTION OF THEOPHYLLINE PREPARATIONS FROM THE STOMACH OF RABBITS<sup>a</sup>

	Time			
	30 Minutes	1 Hour	3 Hours	7 Hours
Theophylline	110 ± 39	169 ± 83	347 ± 70	521 ± 96
Calcium theophyllinate	112 ± 52	145 ± 57	291 ± 49	473 ± 45
Aminophylline	127 ± 62	178 ± 67	404 ± 105	474 ± 64
Aminophylline-Al(OH) <sub>3</sub> gel	106 ± 21	170 ± 54	386 ± 91	579 ± 115
Aminophylline-Al(OH) <sub>3</sub> -ethyl aminobenzoate	109 ± 57	174 ± 81	313 ± 76	472 ± 106
Choline theophyllinate	123 ± 38	243 ± 61	370 ± 56	519 ± 86

<sup>a</sup> Theophylline mcg /100 cc. of blood

TABLE II—BLOOD THEOPHYLLINE LEVELS OBTAINED FROM ABSORPTION OF THEOPHYLLINE PREPARATIONS FIRST FROM THE STOMACH AND THEN FROM THE REMAINDER OF THE GASTROINTESTINAL TRACT OF RABBITS<sup>a</sup>

	Time			
	30 Minutes	1 Hour	3 Hours	7 Hours
Theophylline	115 ± 75	172 ± 93	318 ± 59	587 ± 180
Calcium theophyllinate	57 ± 28	161 ± 79	414 ± 79	580 ± 119
Aminophylline	93 ± 49	144 ± 35	428 ± 54	688 ± 149
Aminophylline-Al(OH) <sub>3</sub> gel	93 ± 30	148 ± 31	282 ± 20	560 ± 182
Aminophylline-Al(OH) <sub>3</sub> -ethyl aminobenzoate	135 ± 23	340 ± 220	424 ± 182	827 ± 216
Choline theophyllinate	157 ± 36	319 ± 14	654 ± 127	724 ± 130

<sup>a</sup> Theophylline mcg /100 cc. of blood

TABLE III.—BLOOD THEOPHYLLINE LEVELS OBTAINED FROM ABSORPTION OF THEOPHYLLINE PREPARATIONS FIRST FROM THE STOMACH AND THEN FROM THE REMAINDER OF THE GASTROINTESTINAL TRACT OF DOGS<sup>a</sup>

	Time					
	30 Minutes	1 Hour	2 Hours	3 Hours	4 Hours	5 Hours
Theophylline	420 ± 41	508 ± 110	1044 ± 190	1238 ± 258	1591 ± 343	1715 ± 345
Calcium theophyllinate	507 ± 383	715 ± 465	1145 ± 398	1483 ± 531	1618 ± 385	1495 ± 174
Aminophylline	545 ± 88	905 ± 134	1569 ± 408	1976 ± 538	2320 ± 450	2449 ± 418
Aminophylline-Al(OH) <sub>3</sub> gel	530 ± 86	1045 ± 96	1215 ± 432	1549 ± 582	1670 ± 626	1662 ± 604
Aminophylline-Al(OH) <sub>3</sub> -ethyl aminobenzoate	760 ± 400	1222 ± 456	1227 ± 462	1998 ± 755	2061 ± 880	1962 ± 826
Choline theophyllinate	654 ± 225	853 ± 298	1410 ± 248	1728 ± 294	1955 ± 429	2085 ± 403

<sup>a</sup> Theophylline mcg /100 cc. of blood.

alone; the subsequent levels are obtained from the entire gastrointestinal tract over a period of six and one-half hours. The results here, as in Table I, show a wide spread of values, and no group is consistently superior.

We repeated this same experiment using dogs, varying only in the increased dose of medication and obstruction of the stomach for a shorter period. The clamp at the distal end of the stomach was removed at the end of one hour and the experiment allowed to continue for five hours. There is likewise significant absorption from the dog stomach during the first hour which continues from the remainder of the gastrointestinal tract. The results, as shown for the six drugs in Table III, appear to fall in the same pattern as the preceding experiments (Tables I and II).

### DISCUSSION

In previous experiments with hospitalized patients we showed that aminophylline tablets were readily absorbed. By the first hour all persons so tested had a definite blood theophylline level.

However, with this dosage given the values ranged from 100 to 500 mcg. per ml. of blood.

In this experiment with rabbits and dogs there is a similarly satisfactory degree of absorption with all drugs tested. There is also the wide spread of levels within the groups using the same brand of preparation.

There is no apparent superiority when the preparations under test are given with the same theophylline content. Clinically it has been observed that the various combinations and modifications of aminophylline give no great superiority over the basic material. No real therapeutic advantage can be claimed for the various additives.

### REFERENCES

- (1) Schack, J. A., and Waxler, S. H., *J. Pharmacol. Exptl. Therap.*, **97**, 283(1949).
- (2) Waxler, S. H., Menzies, J., Enger, M., and Clewe, E. R., *Stanford Med. Bull.*, **10**, 313(1952).
- (3) Waxler, S. H., and Moy, H. B., *Ann. Allergy*, **10**, 179 (1952).
- (4) Waxler, S. H., and Moy, H. B., *J. Allergy*, **22**, 434 (1951).
- (5) Waxler, S. H., and Schack, J. A., *J. Am. Med. Assoc.*, **143**, 736 (1950).

## Notes

### Note on the Alkaloids of *Methysticodendron amesianum*\*

By IRWIN J. PACHTER and ALICE F. HOPKINSON

HIGH in the Colombian Andes, in the geographically isolated valley of Sibundoy, grows a rare and beautiful narcotic tree employed by the Inga and Kamsá Indians in their witchcraft. The tree, *Methysticodendron amesianum*, was described in 1955 by Professor R. E. Schultes (1) who held that it is a member of a hitherto undescribed genus of the *Solanaceae*. The Indians classify the plant together with other intoxicating solanaceous plants of the region, but find that it is even more potent and dangerous than the tree species of *Datura*. A description of the intoxication indicates that the principles are alkaloids of the tropane series (1).

In the hope that this unexplored genus might yield new tropane alkaloids of greater potency and utility than those known, supplies of the plant were obtained from Colombia for study.<sup>1</sup>

Prolonged extraction with hot ethanol caused extensive decomposition of the alkaloidal principles.

It is of interest, in this connection, that the Indians prepare infusions of the leaves with cold water, heating slightly just before administration. The liquid is never allowed to boil (1).

Continuous extraction with cold ethanol followed by concentration *in vacuo* and standard processing yielded crude alkaloidal material in 0.3% yield. Paper chromatography showed the bases to consist of one very major alkaloid and three minor alkaloids.

The total alkaloid mixture was tested pharmacologically and was found to produce biological effects which were practically identical, both quantitatively and qualitatively, with those produced by *l*-scopolamine.

Treatment of the mixture with picric acid in methanol yielded *l*-scopolamine picrate from which the pure base was generated. The alkaloid was identified through comparison of its infrared spectrum and its hydrobromide, hydriodide and methiodide derivatives with those of authentic *l*-scopolamine.

From the picrate mother liquors, mixed residual bases were isolated. Paper chromatography indicated that the same four bases were present, *l*-scopolamine and one other now being predominant.

The second predominant alkaloid proved to be atropine. It was not isolated but was identified through comparison of its *R<sub>f</sub>* values in three solvent systems with those of atropine.

The two minor bases were not identified.

\* Received July 8, 1960, from the Research and Development Division, Smith Kline and French Laboratories, Philadelphia 1, Pa.

<sup>1</sup> Presented at the Conference of Plant Chemists, Bethesda, Md., October 17, 1958.

The authors are grateful to Professor R. E. Schultes of Harvard University, Cambridge, Mass., and Dr. J. M. Idrobo of Bogota, Colombia, for supplies of authentic plant material.

Note added in proof. After this manuscript was submitted, Professor Schultes informed us that a thesis on *Methysticodendron amesianum* has been written by J. F. Thekkumkall of the Universidad Nacional de Colombia.



dissolve readily, but the hydrochlorides or sulfates of the alkaloids and sugars dissolve quite slowly. In these cases the flask was shaken in a mechanical shaker for a few hours, and if necessary warmed to 50–60° in order to achieve complete solution. The solution was then brought to volume and mixed. Because of their lower viscosity, solution was more rapid in pyridine and the amino alcohols than in quinoline or isoquinoline. The solutions should be protected from strong light during preparation and handling, and should not be allowed to stand for longer than twenty-four hours before tak-

ing the rotation, otherwise oxidation of the solvent with subsequent darkening, will result.

The result of this study is presented in Table I. Of the 32 compounds and 10 solvents studied, at least twelve of the solutes exhibited the highest rotation in quinoline, and five in isoquinoline. The effect of quinoline on the rotation of ascorbic acid and of colchicine is most striking. The rotation of the former is magnified fourteenfold as compared with that in water, while the high specific rotation of colchicine (about 425°) in aqueous solution is reduced, in quinoline, to less than one-twentieth of this value.

---

## Communication to the Editor

---

### Color Selection for Uncoated Tablets\*

Sir:

Selection of acceptable colors for uncoated tablets is difficult for several reasons: (a) It is difficult to obtain uniquely colored tablets unless two or more colorants are blended. This blending process is rather haphazard and very time-consuming. (b) Many of the approved colorants do not exhibit desirable light stability. (c) The status of several commonly used FD&C and D&C dyes and pigments is uncertain.

With these limitations in mind, it was desirable to prepare a tablet color bank using light-stable pigments whose status is unquestioned. Since red, blue, and yellow are the primary colors, it is theoretically possible to obtain any desirable hue by blending these colors. Thus, it was reasoned that three colored granulations could be used to prepare tablets of any desired hue. Because only light-stable pigments would suffice, the following D&C colors were selected: Blue No. 6, Red No. 35, and Yellow No. 11 (1).

Three separate wet granulations of these primary colors were prepared containing 0.1 per cent blue, 0.1 per cent red, and 0.2 per cent yellow pigments. Sieve analyses showed that 92 per

cent of these colored granulations passed through a 100-mesh screen (2).

A number of small batches of tablets containing varying ratios of two of the three primary granulations were prepared. Pigment ratios varied as follows: yellow 1 part:red  $\frac{1}{43}$  to 4 parts, red 1 part:blue  $\frac{1}{24}$  to 4 parts, and yellow 1 part:blue  $\frac{1}{43}$  to  $2\frac{1}{2}$  parts. All of these combinations gave evenly colored tablets with different hues. Tablets with less intense color were also prepared by diluting the blended granulations with noncolored ones. In some cases the dilution was 1:20.

This approach is useful for preparing a rainbow of pastel colored tablets. When a hue has been selected, subsequent tablet batches can be prepared by combining the pigments in the desired quantities and granulating in the conventional manner.

(1) Tucker, S. J., Nicholson, A. E., and Engelbert, H. *This Journal*, 47, 849 (1958).

(2) Tucker, S. J., and Hayes, H. M., *ibid.*, 48, 362 (1959).

HENRY C. CALDWELL

Smith Kline and French Laboratories  
Philadelphia, Pa.

\*Received August 4, 1960

## Book Notices

*The Neurochemistry of Nucleotides and Amino Acids.* Edited by ROSCOE O. BRADY and DONALD B. TOWER. John Wiley & Sons, 440 Fourth Ave., New York 16, N. Y., 1960. xii + 292 pp. 14.5 × 23 cm. Price \$10.

This book covers the symposium of the Section on Neurochemistry of the American Academy of Neurology held April 24-25, 1958. Papers are followed by discussions, with a general summary discussion. Author and subject indexes are appended.

*Staining Procedures.* 2nd ed. Revised by H. J. CONN, MARY A. DARROW, and VICTOR M. EMMEL. The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1960. xii + 289 pp. 15 × 23 cm. Paperbound. Price \$5.

This is an authoritative compilation of staining procedures used by the Biological Stain Commission, University of Rochester Medical Center. The procedures are not intended to be considered as standard or official but they are presented in a standardized style for the convenience of users. A given procedure is frequently one of a number of ways of obtaining good staining results. However, one can rely on the tested methods described. A bibliography and subject index are appended.

*Nucleoproteins.* Edited by R. SROOPS. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. 364 pp. 16 × 24.5 cm. Price \$10.50.

This book records the proceedings of the eleventh Solvay conference on chemistry held June 1-6, 1959, at Brussels, Belgium. Paper titles are: Le rôle biologique des acides nucléiques, Molecular structure of deoxyribonucleoproteins, Constitution of histones, Les propriétés physiques de l'acide désoxyribonucléique en solution, Heterogeneity of nucleic acids and effects of chemical and physical agents, Nucleic acids of microorganisms, Biosynthesis of ribonucleic acid, Formation of helical polynucleotide complexes, Structure of RNA containing viruses, and Chemical synthesis of polynucleotides. Each paper is discussed and a general discussion is included. No index.

*Radioactivity for Pharmaceutical and Allied Research Laboratories.* Edited by ABRAHAM EDELMANN. Academic Press, Inc., 111 Fifth Ave., New York 3, N. Y., 1960. xii + 171 pp. 13 × 20.5 cm. Price \$8.

This little book includes the papers presented at a symposium sponsored by Nuclear Science and Engineering Corporation. Subjects discussed include: Effects and utilization of ionizing radiation; Principles, methods, and areas of usefulness of radioactivity in the pharmaceutical and allied sciences; assays, product development, drug absorption, and excretion; and Radiobiochemistry in the pharmaceutical industry. The isotope development program of the Atomic Energy Commission is reviewed.

*Advances in Pest Control Research.* Vol. 3. Edited by R. L. METCALF. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. vii + 448 pp. 15.5 × 23 cm. Price \$14.50.

This third volume of the series continues the excellent reporting in this special field. Volumes I and II were reviewed, respectively, in *THIS JOURNAL*, 47, 230(1958), and 48, 258(1959).

*The Search for New Antibiotics.* By G. F. GAUSE. Yale University Press, New Haven, Conn., 1960. x + 97 pp. 14 × 21.5 cm. Price \$4.75.

This second volume in the Trends in Science program is based on a lecture in December 1959 by G. F. Gause, Director of the Institute of Antibiotics of the Academy of Medical Sciences, Moscow, U. S. S. R. Principles which are known and represent the scientific basis of the search for new antibiotics are given by Gause as: A general microbiological approach to the geography and ecology of microorganisms producing antibiotics; Early classification of microorganisms in the screening program; and Development of new test organisms for rapid detection of specific selective inhibition of some biochemical mechanisms, as illustrated by the search for potential anticancer products.

*British National Formulary 1960.* Alternative Edition. The British Medical Association and the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, England, 1960. Obtainable in the U. S. from The Rittenhouse Bookstore, 1706 Rittenhouse Sq., Philadelphia, Pa. 292 pp. 10.5 × 16.5 cm. Price \$1.75, interleaved \$2.25.

This "alternative" edition of the B. N. F. 1960 contains much of the same information as does the standard edition; but the arrangement of the drug section in this edition is based on a pharmacological classification. These books are intended as guides in prescribing.

*An Introduction to Pharmacology.* By J. J. LEWIS. E. and S. Livingstone Ltd., Edinburgh and London, 1960. Exclusive U. S. agents, The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md. xii + 326 pp. 14 × 21.5 cm. Price \$11.

This book stresses the site, mode, and type of action of drugs, with attention to chemical structure and action relationships. It indicates the lack of knowledge of modes of action of some of the most widely used drugs and develops the text material to inspire further research. This is to be expected from an author who is still actively engaged in research in this field. The text material is presented in an interesting manner, omitting details available in more comprehensive publications. It is a good review and reference book.

*Aids to Biochemistry.* 5th ed. By S. P. DATTA and J. H. OTTAWAY. Baillière, Tindall and Cox, London, 1960. Exclusive U. S. agents, The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md. viii + 266 pp. 10 X 16 cm. Price \$3.75.

This compact book is based on the lecture course in biochemistry for preclinical medical students at University College, London.

*Biochemistry of Steroids.* By ERICH HEFTMANN and ERICH MOSETTIG. Reinhold Publishing Corp., 430 Park Ave., New York 22, N. Y., 1960. xi + 231 pp. 15 X 23 cm. Price \$5.75 college, \$6.90 trade.

This book is designed as a brief introduction to the biochemistry of steroids in which organic chemistry is kept as simple as possible. It is intended for students and for organic chemists in the pharmaceutical industry. Arranged for understanding of biogenetic relations, the chapter heads are: Cholesterol, Sterols, Vitamin D group, Steroid sapogenins and alkaloids, Cardiac glycosides, Bile acids, Progesterone, Corticosteroids, Androgens, and Estrogens. Each class is presented in monograph style. An extensive bibliography and an index are appended.

*The Metabolism of Cardiac Glycosides.* By S. E. WRIGHT. Charles C Thomas, 301-327 East Lawrence Ave., Springfield, Ill., 1960. viii + 86 pp. 15 X 23 cm. Price \$4.75.

Much of the information in this book has been obtained since the development of sensitive methods of analysis, paper chromatographic techniques, and the availability of radioactive glycosides. The text material covers the chemical nature, structure, and pharmacological action of cardiac glycosides; their absorption, distribution, and excretion, and their metabolites.

*Précis de Chimie Générale et de Chimie Minérale.* Vol. 2. By L. DOMANGE. Masson et Cie., 120, boulevard Saint-Germain, Paris 6<sup>e</sup>, France, 1959. viii + 317 pp. 16 X 21 cm. Price 27 NF.

This second volume (in French) considers the elements of general chemistry (thermochemistry, equilibria, etc.), generalities of metals and their compounds, and descriptions of the metals and their compounds. Volume one was reviewed in THIS JOURNAL, 48, 426(1959).

*The Pharmaceutical Pocket Book.* 17th ed. The Council of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, 17 Bloomsbury Square, London, England, 1960. Obtainable in the U. S. from The Rittenhouse Bookstore, 1706 Rittenhouse Square, Philadelphia, Pa. xii + 576 pp. 12 X 18.5 cm. Price 30s.

This edition, the seventeenth, of the book of general information for pharmacists is the first revision under the new (1959) Department of Pharmaceutical Sciences of the Pharmaceutical Society of Great Britain. The text material reflects the modern conditions in this field.

*General Chemistry—Laboratory Manual.* By GARTH L. LEE and HARRIS O. VAN ORDEN. W. B. Saunders Co., West Washington Square, Philadelphia 5, Pa., 1960. v + 217 pp. 22 X 28 cm. Paperbound. Price \$3.75.

This manual of laboratory experiments is designed for students of general chemistry. Although no relating statement is made, the coverage of material is as broad as that in the textbook on the same subject by the authors of the manual.

*Cahiers de Synthèse Organique.* Vol. 6. Méthodes et Tableaux D'Application. Edited by LEON VELLUZ. Masson et Cie., 120, Boulevard Saint-Germain, Paris VI<sup>e</sup>, France, 1960. 417 pp. 16 X 22 cm. Price 120 NF.

This volume considers: Rearrangement of carbon structures, Functional migration between carbons, Functional migration between heteroatom and carbon, and Migration of carbon radicals between heteroatoms. Each section starts with a tabulation of chemical structures involved. This is followed by discussion of principles, mechanisms, applications, and reactions. A tabulation of functional groups and molecular arrangements created by degradation or transposition of compounds considered in vols. 5 and 6 is appended.

*Documenta Geigy.* Scientific Tables. 5th ed. with suppl. S. Karger, Basle, Switzerland, and New York, N. Y., 1959. Available in the U. S. from Albert J. Phiebig, P. O. Box 352, White Plains, N. Y. 547 pp. 16 X 23.5 cm. Price including postage and exchange \$14.45.

This is the fifth edition of data compiled to provide the physician and researcher with basic scientific data in the fields of medicine, biology, chemistry, physics, and mathematics. Much useful information is concisely presented, much of it in tabulations. The table of usual and maximal doses is not sufficiently inclusive because it omits drugs that are in the N. F. Thus, it includes British drugs from the British Pharmacopoeia "or the British Pharmaceutical Codex"; however, the U. S. drugs are U. S. P. but not "or N. F." Since doses are given as they appear in the B. P. or B. P. C., U. S. P., P. 1. (International Pharmacopoeia), etc., a complete comparison is not presented. A supplement covers constituents of living matter, metabolism, synthesis of cell constituents, and detoxication mechanisms. Included are nomograms for assessing body surface areas of children and adults. This is useful in the determination of therapeutic dosage.

*Encyclopaedia of Microscopic Stains.* By EDWARD GURR. The Williams & Wilkins Co., 428 East Preston St., Baltimore 2, Md., 1960. xi + 498 pp. 15 X 24.5 cm. Price \$18.50.

This is a reference book and a laboratory guide on the applications, structures, composition, molecular weights, and properties of most of the important dyes and other substances used for staining microscopic tissue preparations. The major portion of the book (421 pages) is an alphabetical monograph arrangement of stains, indicators, etc. The appended general index is helpful.

JOURNAL OF THE  
AMERICAN PHARMACEUTICAL  
ASSOCIATION

VOLUME 49

OCTOBER 1960

NUMBER 10

A Kinetic Study of Barbitol Degradation in an  
Ammonia Buffer System\*

By J. E. GOYAN, Z. I. SHAIKH, and J. AUTIAN

The kinetics of the degradation of barbitol in an ammonia buffer have been studied. The various species taking part in the parallel reactions leading to decomposition have been taken into account by means of equilibrium constants and, from this, the various second-order rate constants have been calculated. A possible mechanism for the reaction corresponding to known amide hydrolysis mechanisms is postulated.

The total ionic strength was adjusted to 1.0 with potassium chloride in each case. The samples were sealed in 10-cc ampuls and kept in a constant temperature water bath at 40, 50, 60, and  $65 \pm 0.1^\circ$ .

The reaction followed a pseudo first-order course since the log of the absorbance plotted vs. time was a straight line. Table I gives the pseudo first-order rate constants for the different samples, determined by the method of least squares.

THE DECOMPOSITION of barbiturates in aqueous solutions has been recognized for many years (1). The various intermediates have been postulated and isolated (1-4). The rate studies, however, have been of a semiquantitative nature and it was felt that a quantitative study of the kinetics of the decomposition was needed.

## EXPERIMENTAL

**Materials and Apparatus.**—Barbitol, U. S. P. grade, was recrystallized before use (m. p. 189-190°), all other chemicals were reagent grade. A Beckman model DU spectrophotometer and a Leeds and Northrup model 7664 pH meter were used.

**Assay.**—The concentration of barbitol was followed by determining the absorbance of a 2-cc. sample of the experimental solution, diluted to 100 cc. with ammonia buffer (pH 9.5), at 239 m $\mu$ . The spectrophotometric method was chosen since it is rapid and the products of the degradation do not interfere with the determination (2, 5).

**Concentration of Barbitol as a Function of Time.**—Samples of barbitol (0.1% w/v final concentration) were made up at four different pH values (approx pH 8.5, 9.0, 9.5, and 10) using four different concentrations of ammonium chloride for each pH value

## DISCUSSION

In order to separate the different factors in the degradation, it is necessary to take into account the different forms which are present in an aqueous solution of a barbiturate. Calculation of the concentrations of the different forms shows that only the unionized and first ionic forms are important at pH values between 8 and 10.<sup>1</sup>

The rate of degradation of the barbiturate, therefore, is dependent on the concentration of these two forms and probably the amounts of acid and base present. This expectation may be formulated as follows: let  $x$  = unionized barbiturate,  $y$  = ionized barbiturate,  $z$  = total barbiturate,  $H^+$  = hydronium ions,  $OH^-$  = hydroxide ions,  $A$  = acidic form of the buffer,  $B$  = basic form of the buffer,  $-v$  = rate of disappearance of various forms,  $k$  = second-order rate constants for the various possible reaction paths,  $[ ]$  denote concentration and  $( )$  denote activity,  $f$  = activity coefficient of the various forms, and  $K$  = pseudo first-order rate constant. Then

<sup>1</sup> By writing out the different equilibria taking place in the solution and using the principle of electrical neutrality, it is possible to get three equations in the three unknown forms of the barbiturate. Solving, one obtains for the fraction in the second ionic form

$$B = \frac{K_1 K_2}{K_1 K_2 + [H^+]^2 + K_1 [H^+]}$$

Using the approximate value of  $K_2$  given by Butler, *et al.* (6), one finds that the fraction is less than 0.01 at pH 10.

\* Received December 7, 1959, from the College of Pharmacy, University of Michigan, Ann Arbor.  
Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

TABLE 1—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE DEGRADATION OF BARBITAL

NH <sub>4</sub> <sup>+</sup> , M	40° C		50° C		60° C		65° C	
	pH, 25°	K, days <sup>-1</sup>	pH, 25°	K, Days <sup>-1</sup>	pH, 30°	K, Days <sup>-1</sup>	pH, 30°	K, Days <sup>-1</sup>
0 1704	8 60	0 97 × 10 <sup>-2</sup>	8 60	2 69 × 10 <sup>-2</sup>	8 50	7 42 × 10 <sup>-2</sup>	8 60	1 13 × 10 <sup>-1</sup>
0 3408	8 60	1 01 × 10 <sup>-2</sup>	8 60	2 81 × 10 <sup>-2</sup>	8 50	7 84 × 10 <sup>-2</sup>	8 60	1 14 × 10 <sup>-1</sup>
0 5112	8 60	1 03 × 10 <sup>-2</sup>	8 60	3 01 × 10 <sup>-2</sup>	8 50	8 17 × 10 <sup>-2</sup>	8 60	1 19 × 10 <sup>-1</sup>
0 6816	8 60	1 09 × 10 <sup>-2</sup>	8 60	3 10 × 10 <sup>-2</sup>	8 50	8 32 × 10 <sup>-2</sup>	8 60	1 24 × 10 <sup>-1</sup>
0 1292	9 12	1 20 × 10 <sup>-2</sup>	9 12	3 38 × 10 <sup>-2</sup>	9 00	9 60 × 10 <sup>-2</sup>	9 10	1 45 × 10 <sup>-1</sup>
0 2584	9 12	1 24 × 10 <sup>-2</sup>	9 12	3 46 × 10 <sup>-2</sup>	9 00	9 82 × 10 <sup>-2</sup>	9 10	1 52 × 10 <sup>-1</sup>
0 3876	9 12	1 31 × 10 <sup>-2</sup>	9 12	3 58 × 10 <sup>-2</sup>	9 00	1 01 × 10 <sup>-1</sup>	9 10	1 60 × 10 <sup>-1</sup>
0 5168	9 12	1 36 × 10 <sup>-2</sup>	9 12	3 77 × 10 <sup>-2</sup>	9 00	1 04 × 10 <sup>-1</sup>	9 10	1 61 × 10 <sup>-1</sup>
0 0731	9 60	1 39 × 10 <sup>-2</sup>	9 60	3 86 × 10 <sup>-2</sup>	9 50	1 05 × 10 <sup>-1</sup>	9 60	1 63 × 10 <sup>-1</sup>
0 1462	9 60	1 42 × 10 <sup>-2</sup>	9 60	4 07 × 10 <sup>-2</sup>	9 50	1 08 × 10 <sup>-1</sup>	9 60	1 69 × 10 <sup>-1</sup>
0 2193	9 60	1 50 × 10 <sup>-2</sup>	9 60	4 35 × 10 <sup>-2</sup>	9 50	1 13 × 10 <sup>-1</sup>	9 60	1 77 × 10 <sup>-1</sup>
0 2924	9 60	1 53 × 10 <sup>-2</sup>	9 60	4 43 × 10 <sup>-2</sup>	9 50	1 16 × 10 <sup>-1</sup>	9 60	1 81 × 10 <sup>-1</sup>
0 0308	10 10	1 78 × 10 <sup>-2</sup>	10 10	4 55 × 10 <sup>-2</sup>	10 00	1 22 × 10 <sup>-1</sup>	10 10	1 86 × 10 <sup>-1</sup>
0 0616	10 10	1 82 × 10 <sup>-2</sup>	10 10	4 67 × 10 <sup>-2</sup>	10 00	1 24 × 10 <sup>-1</sup>	10 10	1 91 × 10 <sup>-1</sup>
0 0924	10 10	1 92 × 10 <sup>-2</sup>	10 10	4 78 × 10 <sup>-2</sup>	10 00	1 28 × 10 <sup>-1</sup>	10 10	1 97 × 10 <sup>-1</sup>
0 1232	10 10	1 99 × 10 <sup>-2</sup>	10 10	4 97 × 10 <sup>-2</sup>	10 00	1 34 × 10 <sup>-1</sup>	10 10	2 05 × 10 <sup>-1</sup>

using the usual formulation for general acid-base catalysis (7)

$$-v_x = k_x^o[x] + k_x^{H^+}[x][H^+] + k_x^{OH^-}[x][OH^-] + k_x^A[x][A] + k_x^B[x][B] \quad (\text{Eq. 1})$$

$$-v_y = k_y^o[y] + k_y^{H^+}[y][H^+] + k_y^{OH^-}[y][OH^-] + k_y^A[y][A] + k_y^B[y][B] \quad (\text{Eq. 2})$$

Preliminary experiments indicated that no degradation of barbitol took place after fifteen days in 0.1 N hydrochloric acid solution at 60°. From these results it may be assumed that the reaction rate constants  $k_x^o$  and  $k_x^{H^+}$  are negligible in comparison with the other constants and thus may be eliminated. If this is the case, then  $k_x^A$  may also be disregarded.

Now  $[x] + [y] = [z]$   
and  $x \rightleftharpoons H^+ + y$

$$K_A = \frac{(H^+)(y)}{(x)} = \frac{[H^+][y]}{[z] - [y]} \frac{f_n + f_y}{f_x} \quad (\text{Eq. 3})$$

since  $f_x \cong 1$ , and defining  $f_n + f_y = f^2$  gives

$$K_A = \frac{[H^+][y]}{[z] - [y]} f^2 \quad (\text{Eq. 4})$$

solving for  $y$

$$[y] = \frac{K_A[z]}{K_A + [H^+]f^2} \quad (\text{Eq. 5})$$

similarly

$$[x] = \frac{[H^+]f^2[z]}{K_A + [H^+]f^2} \quad (\text{Eq. 6})$$

setting;  $-v_x - v_y = -v_z$  and letting  $\alpha = K_A + [H^+]f^2$ , and also noting that  $-v_z/[z] = K$ , and combining

$$\alpha K = k_x^{OH^-}K_w f^2 + k_x^B[H^+][B]f^2 + k_y^oK_A + k_y^{H^+}K_A[H^+] + k_y^{OH^-}K_A[OH^-] + k_y^AK_A[A] + k_y^BK_A[B] \quad (\text{Eq. 7})$$

Further simplification of the above equation may take place if the buffer dissociation is considered

$$K_A' = \frac{(B)(H^+)}{(A)} = \frac{[B][H^+]}{[A]} \frac{f_H + f_A}{f_B} \quad (f_B = 1) \quad (\text{Eq. 8})$$

define then  $K_A^o = [B][H^+]/[A]$ .

Using this information we may rewrite Eq. 7 follows:

$$\alpha K = k_y^oK_A + k_x^{OH^-}K_w f^2 + k_y^{H^+}K_A[H^+] + k_y^{OH^-}K_w K_A[1/H^+] + [k_x^BK_A^of^2 + k_y^AK_A + k_y^BK_AK_A^o[1/H^+]][A] \quad (\text{Eq. 9})$$

This equation shows that a plot of  $K$  vs.  $[A]$  should be a straight line with slope  $m$  and  $y$  axis intercept of  $b$ .

A typical plot is shown for 40° and several pH values, in Fig. 1. In order to interpret the results further it is necessary to find values for the different dissociation constants and activity coefficients.

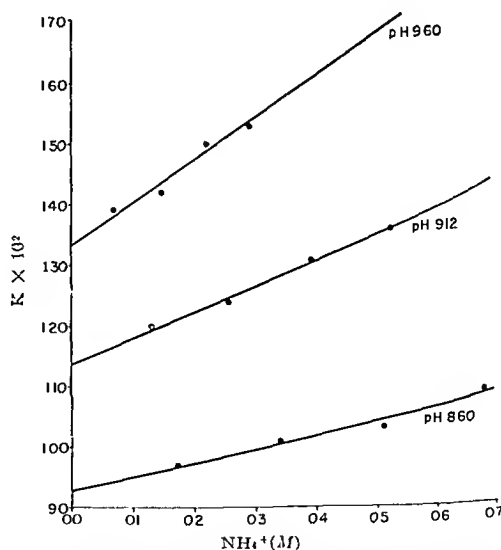


Fig. 1.—Pseudo first-order rate constants plotted versus ammonium ion concentration at 40°.

The dissociation constants for the ammonia buffer system were calculated from (8)

$$pK'_A = \frac{2835.76}{T} - 0.632 + 0.001225T \quad (\text{Eq. 10})$$

the dissociation constants for barbitol by (9)

$$pK_A = \frac{2324.47}{T} - 3.349 + 0.01186 T \quad (\text{Eq. 11})$$

and (10)

$$pK_w = \frac{4470.99}{T} - 6.088 + 0.01706 T \quad (\text{Eq. 12})$$

The determination of the activity coefficients was a more difficult task. Individual ionic activities are, of course, not defined. However, reasonable values for them can be estimated and used (11). The value for  $f_v$  was adopted from the paper by Krahl (12). The activity coefficients for  $[\text{NH}_4^+]$  and  $[\text{H}^+]$  were estimated using the MacInnes assumption (13) and data on the mean ionic activities of ammonium chloride, dilute hydrochloric acid, and water given in Harned and Owen (14). The approach using hydrochloric acid gave a value of 0.88 for  $f_{\text{H}^+}$ . Using water, a value of 0.85 was obtained. We used a value of 0.87 in our work. We assumed a decrease of 0.01 unit to take place between 50 and 60°.

Inspection of Eq. 9 shows that

$$\alpha_{\text{III}} = k_z^B K_A f^2 + k_v^A K_A + k_v^B K_A K_A^0 [1/\text{H}^+] \quad (\text{Eq. 13})$$

and

$$\alpha_B = k_v^B K_A + k_z^{\text{OH}-} K_w f^2 + k_v^{\text{H}^+} K_A [\text{H}^+] + \frac{k_v^{\text{OH}-} K_A K_A^0 [1/\text{H}^+]}{f} \quad (\text{Eq. 14})$$

The hydrogen ion concentration was calculated using Eq. 8. It was solved at 25° to obtain  $C_B$  knowing the pH. Then with known  $C_B$  it was possible to solve for hydrogen ion concentration at the other temperatures, using the appropriate values of  $K_A'$  and activity coefficients.

Equation 14 can be written in the form

$$Y = k_1 + k_2 [\text{H}^+] + k_3 [1/\text{H}^+] \quad (\text{Eq. 15})$$

Attempts to fit this curve by the method of least squares gave a negative value for one of the constants. One constant at a time was then dropped

and the only reasonable fit to the data was obtained from the equation

$$Y = k_1 + k_3 [1/\text{H}^+] \quad (\text{Eq. 16})$$

It was therefore assumed that  $k_v^{\text{H}^+}$  is equal to zero and consequently  $k_v^A$  is also zero. This appears reasonable since  $k_z^{\text{H}^+}$  and  $k_z^A$  are zero. Table II contains the values of  $k_1$  and  $k_3$  calculated by the method of least squares. The values for  $k_v^{\text{OH}-}$  are also calculated and given in Table II. Unfortunately,  $k_1$  contains both  $k_v^B$  and  $k_z^{\text{OH}-}$ , however, it is reasonably certain that  $k_z^{\text{OH}-}$  is much greater than  $k_v^B$ , and  $k_v^B$  is therefore neglected. The values for  $k_z^{\text{OH}-}$  are calculated and given in Table II.

Equation 13 is of the type (after  $k_v^A$  is dropped)

$$Y = k_4 + k_5 [1/\text{H}^+] \quad (\text{Eq. 17})$$

The values of  $k_4$  and  $k_5$  were obtained by the method of least squares and the values of  $k_v^B$  and  $k_z^B$  calculated from these. The values are given in Table III.

Arrhenius type plots of  $\log k$  vs  $1/T$  are shown in Figs. 2-5. The following activation energies are found from these figures:  $E_A(k_z^B) = 16.0 \times 10^3$ ,  $E_A(k_v^B) = 16.2 \times 10^3$ ,  $E_A(k_z^{\text{OH}-}) = 14.9 \times 10^3$ , and  $E_A(k_v^{\text{OH}-}) = 11.4 \times 10^3$ , all in calories per mole.

**Effect of Ionic Strength.**—The effect of ionic strength on a reaction such as this is difficult to predict since there are apparently four different parallel reactions. The final equation may be rewritten in terms of the theory of absolute reaction rates as follows (15)

$$\alpha K = \frac{k_z^{\text{OH}-} K_w}{f} + \frac{2f_{\text{OH}-} f_z}{f^2} + k_z^B [\text{H}^+] [B] + \frac{2f_z f_b}{f^2} + k_v^B K_A [B] \frac{f_{\text{H}^+} f_b}{f^2} + \frac{k_v^{\text{OH}-} K_A [\text{OH}-]}{f} \frac{f_{\text{H}^+} f_{\text{OH}-}}{f^2} \quad (\text{Eq. 18})$$

The activity coefficient ratios for the first three terms may be dropped since the activity coefficient for the activated complex should be approximately equal to the activity coefficient of the numerator

TABLE II—b ANALYSIS

$t, ^\circ\text{C}$	$K_A$	$K_w$	$K_A K_w$	$k_1$	$k_3$	$f^*$
40	$1.66 \times 10^{-8}$	$2.92 \times 10^{-14}$	$4.85 \times 10^{-22}$	$1.90 \times 10^{-10}$	$2.42 \times 10^{-20}$	0.41
50	$2.04 \times 10^{-8}$	$5.48 \times 10^{-14}$	$11.18 \times 10^{-22}$	$6.88 \times 10^{-10}$	$1.03 \times 10^{-19}$	0.41
60	$2.63 \times 10^{-8}$	$9.61 \times 10^{-14}$	$25.27 \times 10^{-22}$	$2.60 \times 10^{-9}$	$3.79 \times 10^{-19}$	0.40
65	$2.95 \times 10^{-8}$	$1.29 \times 10^{-13}$	$38.08 \times 10^{-22}$	$4.42 \times 10^{-9}$	$7.60 \times 10^{-19}$	0.40

$$k_v^{\text{OH}-} = \frac{k_3}{K_w K_A}$$

$t, ^\circ\text{C}$	$k_z^{\text{OH}-}$ (L moles <sup>-1</sup> days <sup>-1</sup> )	$\log k_v^{\text{OH}-}$
40	$4.99 \times 10^1$	1.70
50	$9.23 \times 10^1$	1.97
60	$1.50 \times 10^2$	2.18
65	$1.99 \times 10^2$	2.30

assuming  $k^{\text{OH}-} \gg k_v^B$

$t, ^\circ\text{C}$	$K_w f^2$	$k_z^{\text{OH}-}$ (L moles <sup>-1</sup> days <sup>-1</sup> )	$\log k_z^{\text{OH}-}$
40	$1.20 \times 10^{-14}$	$1.58 \times 10^1$	4.20
50	$2.24 \times 10^{-14}$	$3.07 \times 10^1$	4.49
60	$3.84 \times 10^{-14}$	$6.77 \times 10^1$	4.83
65	$5.17 \times 10^{-14}$	$5.17 \times 10^1$	4.93

$$k_z^{\text{OH}-} = \frac{k_1}{K_w f^2}$$

TABLE III.—*m* ANALYSIS

$t, ^\circ \text{C}$	$K_A$	$K_{oA}$	$k_t$	$k_5$	$f^2$
40	$1.66 \times 10^{-8}$	$9.73 \times 10^{-10}$	$2.72 \times 10^{-11}$	$8.97 \times 10^{-20}$	0.41
50	$2.04 \times 10^{-8}$	$1.82 \times 10^{-9}$	$2.03 \times 10^{-10}$	$4.00 \times 10^{-19}$	0.41
60	$2.63 \times 10^{-8}$	$3.12 \times 10^{-9}$	$4.37 \times 10^{-10}$	$2.28 \times 10^{-19}$	0.40
65	$2.95 \times 10^{-8}$	$4.26 \times 10^{-9}$	$13.85 \times 10^{-10}$	$4.28 \times 10^{-18}$	0.40

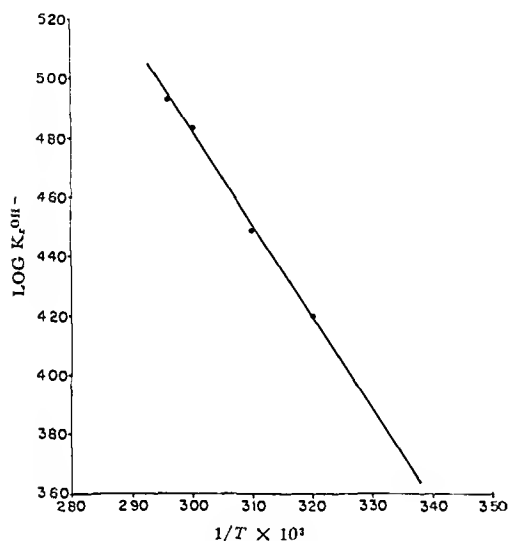
$t, ^\circ \text{C.}$	$K_A K_{oA}$	$(L. \text{ moles}^{-1} \text{ days}^{-1})$	$-\log k_y^B$
40	$1.615 \times 10^{-17}$	$5.55 \times 10^{-3}$	2.26
50	$3.713 \times 10^{-17}$	$1.07 \times 10^{-2}$	1.97
60	$8.389 \times 10^{-17}$	$2.72 \times 10^{-2}$	1.57
65	$12.55 \times 10^{-17}$	$3.41 \times 10^{-2}$	1.47

$t, ^\circ \text{C.}$	$K_{oA} f^2$	$(L. \text{ moles}^{-1} \text{ days}^{-1})$	$-\log k_x^B$
40	$3.98 \times 10^{-10}$	$6.83 \times 10^{-2}$	1.17
50	$7.47 \times 10^{-10}$	$2.72 \times 10^{-1}$	0.57
60	$1.25 \times 10^{-9}$	$3.49 \times 10^{-1}$	0.46
65	$1.70 \times 10^{-9}$	$8.15 \times 10^{-1}$	0.09

$$k_y^B = \frac{k_5}{K_A K^{\circ}A}$$

$$k_z^B = \frac{k_4}{K^{\circ}A f^2}$$

Fig. 2.—Arrhenius type plot for  $k_z^{\text{OH}^-}$ .

The activity coefficient term in  $\alpha$  may be ignored as long as  $K_A$  is larger than  $\text{H}^+$ . As a first approximation the activity coefficients may be substituted with the appropriate value from the Debye Hückel limiting law and the derivative of  $\log K$  with respect to the square root of ionic strength ( $\mu$ ), may be taken. This gives

$$\frac{d \log K}{d \sqrt{\mu}} = \frac{-C_1 10^{-\sqrt{\mu}} + C_2 10 \sqrt{\mu}}{C_1 10^{-\sqrt{\mu}} + C_2 10 \sqrt{\mu}} \quad (\text{Eq. 19})$$

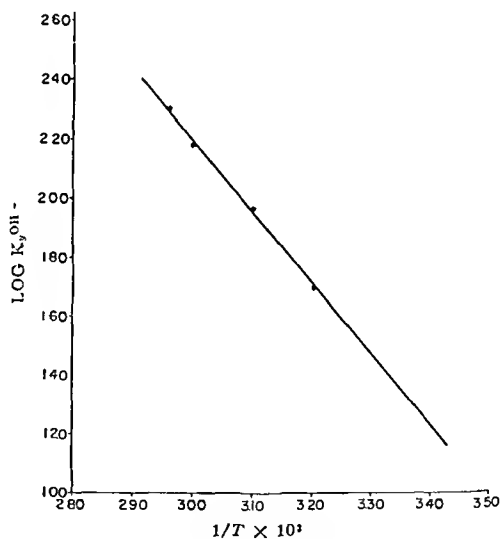
Where  $C_1$  and  $C_2$  are the appropriate lumped constants. At a pH of 9.40 and  $60^\circ$ , using the appropriate constants, a value for the derivative of Eq. 19 was found to be a small negative number. Thus, rate of the reaction should fall off slowly with increases in ionic strength under these particular conditions. The values for this particular experiment were determined and are given in Table IV. Table IV shows that the decrease is indeed slow and the equation is at least approximately correct. The effect of ionic strength is thus qualitatively predictable from this approximate equation.

Mechanism.—There are several interesting as-

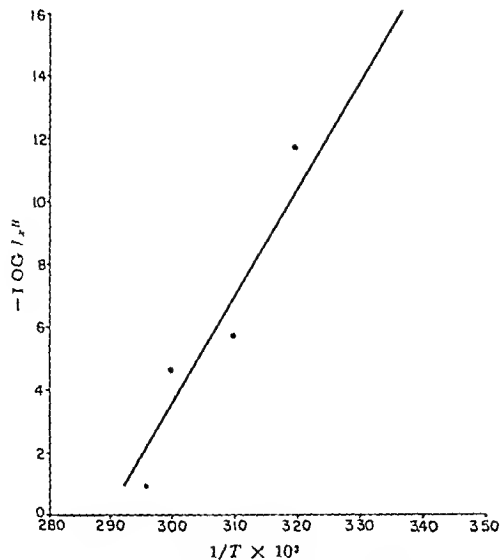
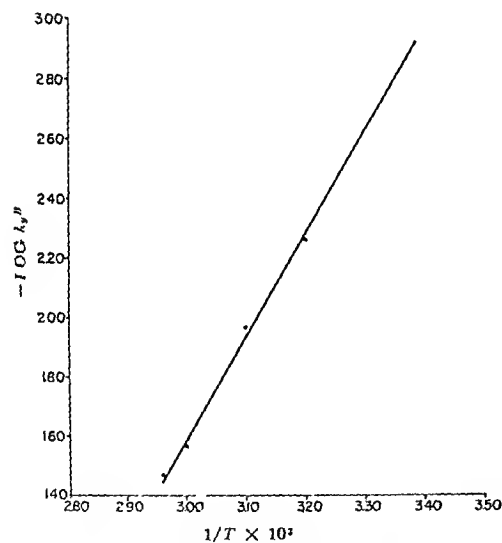
TABLE IV.—THE EFFECT OF IONIC STRENGTH<sup>a</sup>

$\sqrt{\mu}$	$k \times 10^3, \text{hr.}^{-1}$
0.45	4.45
0.630	4.35
0.775	4.28
0.895	4.23
1.000	4.18

<sup>a</sup>  $60^\circ\text{C.}$ , pH 9.40.

Fig. 3.—Arrhenius type plot for  $k_y^{\text{OH}^-}$ .

pects to this degradation. The apparent lack of acid catalysis is surprising in view of the known acidic hydrolysis of many other amides (16, 17). This may be explainable in terms of the very weak basicity of the imide structure. Attempts to titrate barbital in nitromethane with perchloric acid, according to the method of Streuli (18), failed, indicating that barbital is a weaker base than acetamide. Since protonation is probably an important step in the acidic catalysis of amide hydrolysis (16, 19) this weak basicity of the barbital may account for our inability to show acidic catalysis.

Fig 4 — Arrhenius type plot for  $k_x^B$ Fig 5 — Arrhenius type plot for  $k_y^B$ 

The basic hydrolysis of the unionized species can be formulated along known pathways as shown in Fig 6a

The hydrolysis of the ionized species would probably be at the 4 (or 6) position because of the resonance shown in Fig 6b. Hydrolysis at this position would, of course, lead to different products. It is interesting to note in this connection that Fretwurst (4) found that the concentration of diamide (the product of hydrolysis of unionized barbiturate) decreased when the concentration of base was increased. Further work in an attempt to substantiate these mechanisms is in progress.

The catalysis by ammonia is also rather surprising. Perhaps it can be accounted for by visualizing a reaction similar to the ammonolysis of an ester (20)

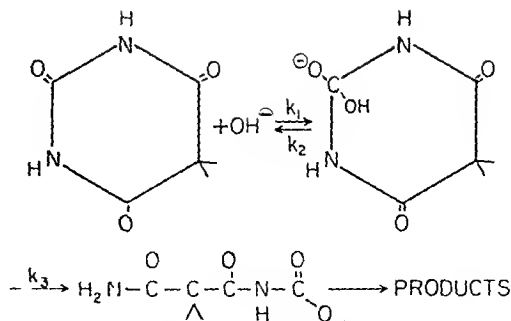


Fig 6a — Hydrolysis mechanism of unionized barbituric acid

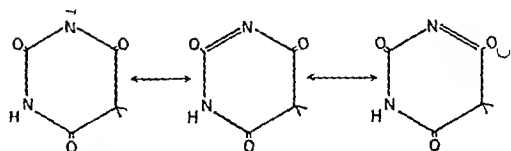


Fig 6b — Resonance forms of the ion

The product of the reaction would be quite unstable and a means of verifying this possibility appears rather difficult.

## SUMMARY

1 The rate of degradation of barbituric acid has been shown to follow a pseudo first-order reaction.

2 The rate constants for the various parallel reactions which take place have been isolated and evaluated.

3 The possible mechanisms of the various reactions are discussed.

## REFERENCES

- (1) Husa W J and Jatul, B B *THIS JOURNAL*, 33, 217 (1944)
- (2) Rotondaro F A *J Assoc Offic Agr Chemists*, 38, 809 (1955)
- (3) Kapadia A J, Goyan J E and Autian, J *THIS JOURNAL*, 48, 407 (1959)
- (4) Fretwurst F *Arzneimittel Forsch*, 8, 44 (1948)
- (5) Jackson, G R, Jr, Weschler, J R and Dannley R L *Anal Chem*, 26, 1661 (1954)
- (6) Butler T C, Ruth J M, and Tucker G F *J Am Chem Soc*, 77, 1486 (1955)
- (7) Laidler, K J *Chemical Kinetics* McGraw Hill Book Co, Inc New York N Y 1950, p 289
- (8) Bates R G and Pinching, G D *J Research Natl Bur Standards*, 42, 419 (1949)
- (9) Manov, G G, Schuette, K E and Kirk F S, *ibid*, 48, 84 (1952)
- (10) Harned H S and Owen, B B, *The Physical Chemistry of Electrolytic Solutions* Reinhold Publishing Corp, New York N Y 1950, p 492
- (11) Bates R G *Electrometric pH Determinations* John Wiley & Sons Inc New York, N Y, 1954, p 39
- (12) Krahl M E, *J Phys Chem*, 44, 449 (1940)
- (13) Bates R G *Electrometric pH Determinations* John Wiley & Sons Inc New York, N Y, 1954, p 48
- (14) Harned H S and Owen B B, *The Physical Chemistry of Electrolytic Solutions* Reinhold Publishing Corp New York N Y 1950, appendix
- (15) Laidler, K J *Chemical Kinetics* McGraw Hill Book Co, Inc New York N Y, 1950, p 123
- (16) Bender, M L, and Ginger, R D, *J Am Chem Soc*, 77, 348 (1955)
- (17) Marcus A D, and Baron S, *THIS JOURNAL*, 48, 85 (1959)
- (18) Streuli C A *J Anal Chem*, 31, 1652 (1959)
- (19) Hine J *Physical Organic Chemistry* McGraw-Hill Book Co, Inc, New York, N Y, 1956, p 266
- (20) *Ibid*, p 295



# Heating and Cooling Rate Coefficients and Related Factors Affecting Procedures for Tablet Shelf Life Prediction\*

By STUART P. ERIKSEN, GEORGE M. IRWIN, and JOSEPH V. SWINTOSKY

Data are presented for the heating and cooling rate coefficients of tablets under the usual conditions of exaggerated temperature hot air oven stability studies. The interrelationships of tablet composition, container dimensions, the drug activation energy and the probability factor of the Arrhenius equation, the storage oven temperature, time of storage, and sample heating and cooling rate coefficients are noted as they influence the programming and results of short term, high temperature thermal degradation studies. Also discussed is the possible influence of other factors within the control of the development pharmacist which may affect the validity of exaggerated temperature thermal drug degradation data used in room temperature shelf life prediction of products.

THIS PAPER describes a quantitative study of the effects of tablet size, coating, bottle size, position in a bottle, and position in a carton on the heating and cooling characteristics of tablets; also discussed are how the tablet heating and cooling characteristics, storage oven operation and temperatures, and other matters pertaining to the programming of an exaggerated temperature stability study may influence the accuracy of shelf life predictions of drugs in tablets.

An awareness of these factors as they affect the heating and cooling behavior of tablets in commercial containers is important because: (a) during the development of new tablet formulations, exaggerated temperature stability studies are usually conducted in containers of size and closure corresponding to those anticipated for the marketed product, (b) exaggerated temperature stability studies usually employ rooms or air ovens at temperatures ranging from 30 to 85° for periods as short as several hours to as long as several years, (c) the best use of the chemical kinetic approach to shelf life prediction requires accurate knowledge of the time and temperature of exposure of each tablet analyzed, (d) analysis of a tableted drug frequently is performed on only a fraction of the tablets in a given bottle or carton, and (e) during transit and storage, pharmaceutical products frequently receive short term exposure to temperatures appreciably higher or lower than normal room temperature.

In an earlier report (1) it was suggested that, in exaggerated temperature studies, errors in

the estimation of product shelf life might result if no consideration was given to the slow heating and cooling processes characteristic of most pharmaceutical solids. It was proposed that an estimate of exaggerated oven temperature storage time errors involved could be calculated on the basis of an "equilibrium temperature time equivalent" (ETTE). This is an expression for the actual storage time of a product at a given temperature corrected for the effects of the finite times required for heating or cooling to an equilibrium temperature.

In our experimental work thermocouples were introduced into individual tablets, and their temperature histories were recorded as a function of time under conditions similar to those used for stability studies. From these data, the heating and cooling characteristics and the ETTE's were determined for the tablets. Using the ETTE concept, calculations were made showing the magnitude of errors that can occur in studies of drug degradation and product shelf life when oven storage times are not corrected for the effects of the heating and cooling processes. Also, calculations were made of the minimum oven storage times beyond which, under conditions similar to these studies, the storage time errors would be relatively insignificant in the calculation of shelf life from short term exaggerated temperature analytical data.

The study of the degree to which various factors influence tablet heating, cooling, and drug degradation, thus influencing the accuracy of chemical kinetic calculations of drug degradation rates, should be helpful in the programming of accelerated studies of product shelf life. Also, these studies give an indication of the effects that temperature extremes may have on packaged pharmaceutical products during such exposure in transit and storage.

\* Received November 25, 1959, from the Research and Development Division of Smith Kline and French Laboratories, Philadelphia 1, Pa.

Presented to the Scientific Section, A. Ph. A., Cincinnati meeting, August 1959.

The authors gratefully acknowledge the assistance of Mr. J. F. Pauls with the statistical portion of this work.

## EXPERIMENTAL

**Thermocouples.**—Polyvinyl coated, 26 gauge solid-wire, iron-constantan thermocouples were used for all measurements of tablet temperatures. Thermocouple junction wires were twisted, welded with a small torch, and then were trimmed short.

**Recording Equipment.**—A Minneapolis-Honeywell "Electronik" potentiometric recorder was used to record temperature as a function of time. The span was so adjusted that temperature was recorded directly in degrees centigrade. All measurements were made using a crushed ice-distilled water reference bath at 0°. Under the above conditions the temperature recording was correct to within 0.5% over a temperature span of 0 to 100°.

**Oven.**—A large (27 cu. ft.), mechanically convected hot-air oven (Hotpack model 1204) was used for all storage studies. The temperature regulation of this oven showed a variation of less than  $\pm 2^\circ$ .

**Tablets.**—Three different tablets were used: (a) Large pink sugar-coated convex vitamin tablets;  $\frac{1}{2}$  inch (1.27 cm) diameter,  $\frac{7}{32}$  inch (0.56 cm) thick, and 0.80 Gm. weight. (b) Small gray sugar-coated convex calcium sulfate tablets;  $\frac{3}{8}$  inch (0.95 cm) diameter,  $\frac{7}{32}$  inch (0.56 cm) thick, and 0.43 Gm weight. (c) Small uncoated convex aspirin tablets;  $\frac{5}{16}$  inch (0.79 cm.) diameter,  $\frac{7}{32}$  inch (0.56 cm.) thick, and 0.30 Gm weight.

The thermocouples were inserted into the tablets drilled holes (No. 60) drilled radially, two-thirds the distance through the tablet. The thermocouples were sealed in the holes with a droplet of deKhotinsky cement (Fig. 1). Several thermocouples were inserted into uncoated tablets during the punching process, using a slotted die (Fig. 2)

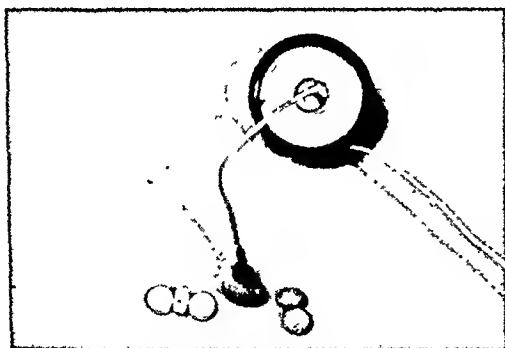


Fig. 1.—Picture showing drilled tablets and placement of thermocouple.

**Containers.**—Standard round amber-glass powder bottles (20,<sup>1</sup> 28,<sup>1</sup> 50, 60, 100, 120, 200, and 250 ml.) with plastic polyvinyl-cardboard lined screw caps were used to hold tablet samples. A one-fourth-inch hole was drilled through the center of the cap and liner to allow the thermocouple wire to be led into the tightly capped bottle. The hole was sealed and the wire and tablet held in place with a cork stopper (Fig. 3). The tablet with the inserted thermocouple was placed in a desired position within the bottle. Then the remaining tablets were poured in and the cap was screwed tight.

<sup>1</sup> These were commercial 5- and 7-dr vials

Standard pasteboard cartons holding 12 bottles each were used for the carton tests. In each case, all 12 bottles contained the same type of tablets as those being measured, and the tablet being studied was located centrally in the bottle. The arrangement of the bottles in the carton is shown in Fig. 4. Reference to Fig. 4 clarifies the designations: corner of carton (COC), side of carton (SOC), and interior of carton (IOC). The temperature of each of the three tablets in the three respective bottles was

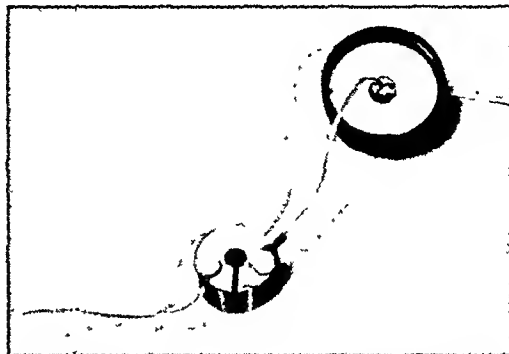


Fig. 2.—Picture showing thermocouple attached to tablet by use of a slotted die during compression



Fig. 3.—Picture showing the drilled cap, stopper holding the thermocouple wire, and tablet placement in the bottle.



Fig. 4.—Picture showing the arrangement of bottles in cartons where thermocouples sealed in tablets were situated in the center of respective bottles

checked and recorded every two minutes with the aid of an automatic switching device (Fig 5). This device was assembled from a 30 r. p. h. synchronous motor, four adjustable cams, and four micro-switches.

In studies on helium-filled containers, bottles filled with tablets were inverted and flushed with helium gas (Matheson Co.) The caps were tightened, and the heating and cooling curves were determined with the bottles inverted.

**Temperature Measurement Program.**—Heating and cooling measurements of temperature *versus* time at three oven temperatures, 45, 60, and 75°, were made on tablets located both centrally and laterally (against the glass) in eight different-sized bottles, and in three bottle positions within the cartons.

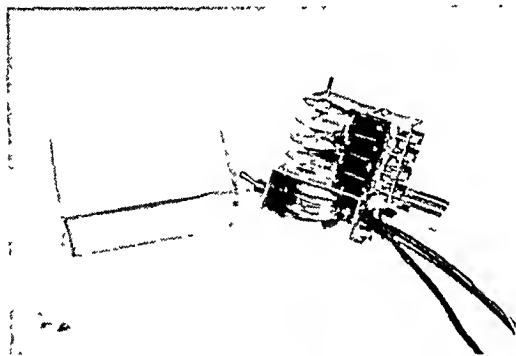


Fig. 5.—Picture showing automatic switching device for monitoring temperatures of tablets within bottles contained in closed pasteboard cartons.

## THEORY AND CALCULATIONS

Ball and others (2, 3, 4) have reported that canned foods heated in ovens or retorts tend to heat over the greater portion of their heating range according to a process which may be expressed as

$$\log(T_{ov} - T) = \frac{-Z_h}{2.303} t + \log(T_{ov} - T_r) \quad (\text{Eq. 1})$$

Cooling may be represented by the equation<sup>2</sup>

$$\log(T - T_r) = \frac{-Z_c}{2.303} t + \log(T_{ov} - T_r) \quad (\text{Eq. 2})$$

The terms  $Z_h/2.303$  and  $Z_c/2.303$  are determined experimentally by plotting  $\log(T_{ov} - T)$  or  $\log(T - T_r)$ , respectively, *versus* time. The slopes of the straight line portions of such plots are numerically equal to  $Z_h/2.303$  and  $Z_c/2.303$ ; where  $Z_h$  and  $Z_c$  are the specific heating and cooling rates (1), respectively. Figure 6 is an illustration of this type of plot.

The heating and cooling rates of the contents of a bottle are related to bottle dimensions and the thermal conductivity, specific heat, and density of the bottle and its contents (2) by the expression

$$Z = \frac{4K}{F_\pi} \times \frac{1}{D^2} \quad (\text{Eq. 3})$$

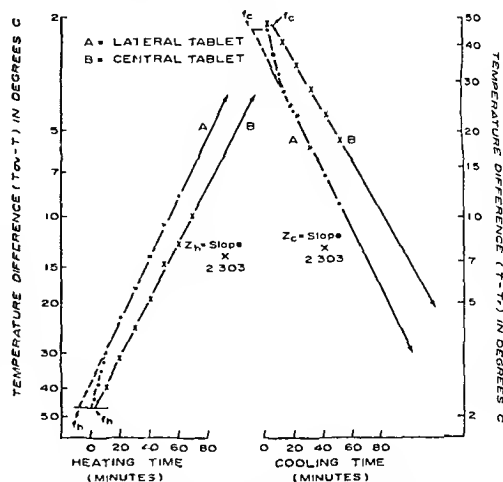


Fig. 6.—Graph showing the relationship of factors  $f_h$ ,  $f_c$ ,  $Z_h$ , and  $Z_c$  in heating and cooling plots for large coated tablets in 250-ml. bottles. Room and oven temperatures were 27 and 75°, respectively. The data obtained from these plots are:

	$Z_h$	$f_h$	$Z_c$	$f_c$
Central	0.023	3	0.020	4
Lateral	0.024	-9	0.023	-9.5

where  $Z$  is either  $Z_h$  or  $Z_c$ , and  $K$  is the diffusivity constant which may be defined by

$$K(\text{Diffusivity}) = \frac{\text{Thermal Conductivity}}{\text{Density} \times \text{Specific heat}} \quad (\text{Eq. 4})$$

Each term on the right of the equality sign in Eq. 4 refers to constants which are characteristics of the bottle plus its contents. The term  $F_\pi$  is a dimensional constant controlled by the ratio of bottle diameter,  $D$ , to height,  $H$ , in the expression

$$\frac{1}{F_\pi} = \frac{1}{2.303} \left[ 5.783 - 2.467 \left( \frac{D}{H} \right)^2 \right] \quad (\text{Eq. 5})$$

This equation is obtained algebraically from the general expression of Williamson and Adams (5) for the sudden heating or cooling of a cylindrical solid.

Plots of  $Z$  *versus*  $1/D^2$  should yield straight lines with a slope of  $4K/F_\pi$  for bottles and containers all having identical diffusivities and the same ratio of  $D/H$  (2). If, as in our studies, dimensions change while the density of the contents remains essentially constant, plots of  $1/D^2 F_\pi$  (essentially dimensional characteristics of the container) *versus*  $Z$  should yield straight lines of slope  $4K$ . The term  $K$  is a thermal characteristic of the bottle plus contents.

To calculate the relative storage time errors resulting from the assumption that all tablets in the container are exposed to temperatures and times of storage corresponding to actual oven temperature and the actual period of oven storage, ETTE's for tablets at the sides and centers of various bottles and cartons have been calculated. The derivation and discussion of these equations have been presented previously (1). The per cent storage time error may be calculated as

<sup>2</sup> Definitions of terms are included in the text only where needed for clarity. A complete glossary is appended.

$$\text{per cent storage time error} = 100 \times \frac{t_2 - \text{ETTE}}{\text{ETTE}} \quad (\text{Eq } 6)$$

where ETTE has been defined as

$$\text{ETTE} = t_0 - t_1 + t_h + (t_c - t_2) \quad (\text{Eq } 7)$$

and  $t_h$  and  $(t_c - t_2)$  as

$$t_h = \frac{A_{10} \int_{t_1}^{t_2} e^{-\Delta H/R(T_{01} - (T_{01} - T_r))e^{-Z_h t}} dt}{k_{01}} \quad (\text{Eq } 8)$$

$$(t_c - t_2) = \frac{A_{12} \int_{t_2}^{t_3} e^{-\Delta H/R(T_r + (T_{01} - T_r))e^{-Z_c t}} dt}{k_{01}} \quad (\text{Eq } 9)$$

The terms  $t_0$ ,  $t_1$ ,  $t_2$ ,  $t_3$  are the time of placement in the oven, the time when the sample reaches oven temperature, the time of removal from the oven, and the time when the sample reaches room temperature again, respectively. Simplified solutions to Eqs 8 and 9 are presented in our previous paper (1)<sup>3</sup>

If a tablet that is under observation does not commence heating or cooling instantly, the time required to begin heating or cooling should be included in the expression for ETTE (Eq 7). To simplify the calculation of these times, we have idealized the heating and cooling curves at the early time periods, and defined the time required to begin heating or cooling according to these simplified curves. As we define it, the time required for a sample to begin an exponential rate of heating or cooling is determined graphically by extrapolating the straight line portion of the logarithmic plot back to the horizontal line which corresponds to the starting temperature difference,  $\log (T_{01} - T_r)$ . The  $f_h$  and  $f_c$  values may be read directly from the intersection of the appropriate  $\log (T_{01} - T_r)$  line and the extrapolated lines. This graphical determination of  $f_h$  and  $f_c$  is illustrated in Fig 6. If the initial tablet heating or cooling rate is relatively fast, the intersection of the lines may occur somewhat before the actual starting time. When the initial rate is relatively slow, the intersection may occur after starting time.

If cognizance is taken of this initial departure from exponential heating and cooling, Eq 7 becomes

$$\text{ETTE} = t_2 - t_1 + f_c + t_h + (t_c - t_2) \quad (\text{Eq } 10)$$

The term  $t_1$  is the difference between the time when the sample is placed in the oven and the time when it reaches oven temperature, so that it includes the factor  $f_h$ . Theoretically,  $t_1$  would be infinite, due to the logarithmic relationship between the temperature and time. For purposes of calculation we can overcome this problem, introducing only a minor

<sup>3</sup> Numerical solutions of the integrals of Eqs 8 and 9 were presented in the previous work in the form

$$t_h = P_h/Z_h = \frac{P_h}{2.303 \times \text{heating slope}} \quad (a)$$

$$(t_c - t_2) = P_c/Z_c = \frac{P_c}{2.303 \times \text{cooling slope}} \quad (b)$$

where values for  $P$  for a room temperature of 25° were given as

Temperature °C	$P_h$	$P_c$
45	2.308	1.241
60	2.328	0.7568
75	2.423	0.5080

In our previous paper (1) Eq 27 on page 699 is in error. The correct equation is shown above as (b).

error, by letting the time when the sample reaches oven temperature be equal to the time when the sample reaches a temperature at which the drug degradation rate is 95% of the value it would have at the equilibrium oven temperature (1). The temperature at which this degradation rate is reached can be calculated readily from the kinetic relationship

$$T_{95\%} = \frac{\Delta H/2.303R}{\Delta H/2.303R \times (1/T_1) - \log 0.95} \quad (\text{Eq } 11)$$

The time to reach this temperature ( $t_1$ ) is found from the equation

$$t_1 = 1/Z_h(2.303[\log(T_{01} - T_r) - \log(T_{01} - T_{95\%})]) + f_h \quad (\text{Eq } 12)$$

or

$$t_1 = \frac{Q}{Z_h} + f_h \quad (\text{Eq } 12)$$

Combining Eqs 10 and 12, the value of ETTE is

$$\text{ETTE} = t_0 + f_c \frac{Q}{Z_c} - f_h + t_h + (t_c - t_2) \quad (\text{Eq } 13)$$

## RESULTS AND DISCUSSION

Dimensions and related information regarding tablets, bottles, and cartons used in this study are given in Table I.

It was noted early in our studies that thermocouples sealed into drilled holes gave data identical with those inserted during compression, so this distinction is not made in the data reported.

**Specific Rates of Heating and Cooling.**—In the heating and cooling experiments, the recordings of the Minneapolis Honeywell recorder were obtained in degrees centigrade, *versus* time in minutes. These data were replotted according to Eqs 1 and 2 as  $\log (T_{01} - T)$  or  $\log (T - T_r)$  *versus* time. The slopes of the straight line portions of these plots were equal to  $Z/2.303$  from which the values of  $Z_h$  and  $Z_c$  are readily determined. Extrapolations of the straight line plots of these data also permit determinations of the intercept values of  $f_c$  and  $f_h$ . Illustrative data for large coated tablets in a 250 ml bottle at an oven temperature of 75° and a room temperature of about 25° are given in Fig 6. In this illustration, a tablet located at the side of the bottle shows a  $Z_h$  and  $Z_c$  of 0.024 and 0.023 min<sup>-1</sup>, respectively. Values for  $f_h$  and  $f_c$  are -9 and -9.5 minutes, respectively. A tablet located at the center of the bottle gives values of  $Z_h = 0.023$  min<sup>-1</sup>,  $Z_c = 0.020$  min<sup>-1</sup>,  $f_h = 3$  minutes, and  $f_c = 4$  minutes.

It is of interest that values of  $Z_h$  for side or centrally located tablets are virtually identical for any given bottle. A similar relationship exists for values of  $Z_c$ . Values of  $f_h$  for side and centrally located tablets are different, and may have opposite signs, as illustrated above. Similarly  $f_c$  values are different under these conditions. Specific heating and cooling rate data for all bottles indicated that  $Z$  values increased as bottle size decreased. Also, the  $Z_h$  for a given type tablet and bottle was always larger than the  $Z_c$ .

TABLE I — PHYSICAL DATA FOR VARIOUS BOTTLES AND CARTONS USED IN TABLET HEATING AND COOLING STUDIES

Bottles					Tablets per Bottle <sup>b</sup>		
Bottle, ml	Height, cm <sup>a</sup>	Diameter, cm	$F_{\pi}$		Large Coated	Small Coated	Small Uncoated
20 (5 dr)	4.8	2.6	0.418		18	44	40
28 (7 dr)	5.3	3.2	0.284		24	57	53
50	5.5	4.2	0.177		51	129	118
60	5.8	4.4	0.161		68	170	157
100	6.8	4.8	0.132		110	270	260
120	7.5	5.4	0.105		135	345	320
200	9.0	6.0	0.083		225	565	530
250	10.0	6.4	0.072		280	705	670

Cartons			
Contents	Length, cm	Width, cm	Height, cm
Twelve 28-ml (7-dr vials)	13.2	9.9	7.1
Twelve 50-ml bottles	17.4	12.8	7.8

<sup>a</sup> The height is the distance from the bottom of the bottle to the neck, excluding the cap and thread  
<sup>b</sup> In all instances bottles were filled to the neck with tablets

Since  $Z_c$  and  $Z_h$  were determined experimentally, and  $D$  and  $F_{\pi}$  are bottle dimensional constants having the values given in Table I, it is possible to utilize Eqs 3 and 4 to obtain plots of  $Z$  versus  $1/D^2 F_{\pi}$ . Such plots are illustrated in Figs 7 and 8. The plots show a close approach to linearity in all heating and cooling studies utilizing containers of 28 ml or larger volumes. The  $Z$  values for the 20-ml bottle show considerable deviation from the straight line toward a lower slope. The linearity of the data for bottles in excess of 28 ml indicates a fairly uniform value for thermal conductivity ( $K$  of Eq 3) in these bottled tablets. The lower diffusivity observed for the 20-ml bottle of tablets suggests that in such small containers, heat transfer has become more dependent upon conduction of the tablet material alone rather than on the tablet-air matrix, as appears to be the case with the larger bottles.

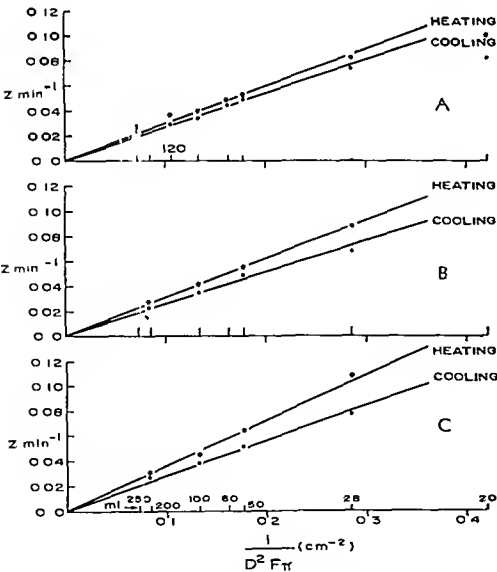


Fig. 7.—Graphs showing the relationship of  $Z_h$  and  $Z_c$  to  $1/D^2 F_{\pi}$  for three tablets, centrally located. A, large coated tablets; B, small coated tablets; C, small uncoated tablets.

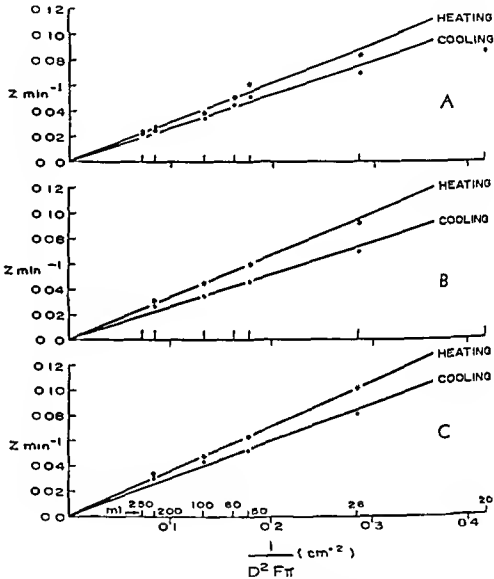


Fig. 8.—Graphs showing the relationship of  $Z_h$  and  $Z_c$  to  $1/D^2 F_{\pi}$  for three tablets, laterally located. A, large coated tablets; B, small coated tablets; C, small uncoated tablets.

Excluding the data for the 20-ml. bottles, the slope of the best straight line, determined mathematically, using the method of least squares, was obtained for plots of  $Z$  versus  $1/D^2 F_{\pi}$  for each heating and cooling experiment performed in these studies. Pooling all of the slopes of these plots in various ways, i. e., all heating slopes versus all cooling slopes, all large coated tablets versus all small coated tablets, etc., an analysis of variance was made to determine the significance of the observed differences of slope resulting from four of the factors with which we were concerned. These factors were (a) heating as compared to cooling, (b) central tablets as compared to lateral tablets, (c) coated as compared to uncoated tablets, and (d) large coated as compared to small coated tablets. Results of this analysis of variance, given in Table II, indicate that only the slopes of

(a) heating as compared to cooling, and (b) coated as compared to uncoated tablets, were significantly different

On the basis of these results, all the  $Z$  versus  $1/D^2F_r$  slopes were divided into four groups, i.e., those derived from (a) heating of coated tablets, (b) heating of uncoated tablets, (c) cooling of coated tablets, and (d) cooling of uncoated tablets. The slope of the best line fitting each of these groups was calculated, along with the standard error, and is given in Table II. Since the standard errors are small, the average values of these slopes in Table II may be used to write specific equations to describe the  $Z_h$  and  $Z_c$  values one would obtain for

these tablets under these stability study conditions for cylindrical bottles ranging in size from 28 to 250 ml. Substituting these average slope values for  $4K$  in Eq. 3, the following equations may be obtained:

$$Z_h(\text{coated tablet}) = 0.3114(1/D^2F_r) \quad (\text{Eq. 14})$$

$$Z_c(\text{coated tablet}) = 0.2608(1/D^2F_r) \quad (\text{Eq. 15})$$

$$Z_h(\text{uncoated tablet}) = 0.3628(1/D^2F_r) \quad (\text{Eq. 16})$$

$$Z_c(\text{uncoated tablet}) = 0.2913(1/D^2F_r) \quad (\text{Eq. 17})$$

These equations were used to assign average  $Z$  values to the 28- to 250-ml bottles employed in this study. These values are recorded in Table IIA. It should be noted that these  $Z$  values are independent of the oven temperatures.

The results summarized in Tables II and IIA indicate that (a) the specific heating and cooling rates ( $Z$  values) for a given type tablet and bottle under these stability study conditions differ by about 20%. The  $Z$  values for heating are always greater (b) Within the tablet size range studied, specific heating and cooling rates are not influenced significantly by tablet dimensions. (c) The specific heating and cooling rates of tablets are the same for those located at the center or side of the bottle. (d) The uncoated tablets heat and cool significantly faster than similar coated ones. These  $Z$  values differ by about 15%. (e) Specific heating and cooling rate coefficients are related directly to the thermal conductance and inversely to the product of density, specific heat, cylindrical bottle diameter squared, and the dimensional constant,  $F_r$ , for each bottle of tablets considered.

Equations 1-4 and 14-17 do not suggest why specific heating and cooling rates should differ significantly, since thermal conductance and diffusivity should be the same for both heating and cooling. These significant differences are probably due to the conditions under which the heating and cooling studies were performed. In conformance with the usual procedure for performing exaggerated temperature stability studies, during the oven heating stage the bottled tablets are exposed to a rapidly moving air atmosphere which is actuated by a blower fan, however, during the cooling phase, bottled tablets are removed from the oven and allowed to stand in relatively still room air. It is very likely that the specific heating and cooling rates of a given bottle of tablets would become identical if, after heating in a blower oven, they were transferred to an identical blower oven with air being blown at room temperature.

TABLE II—SLOPE VALUES OBTAINED FROM  $Z$  Versus  $1/D^2F_r$  PLOTS

Division of Data	Slope	Results of Analysis of Variance on Observed Slope Differences
All heating data <sup>a</sup> versus	0.3295	Significant at $P = 0.01$
All cooling data	0.2729	
All central data <sup>a</sup> versus	0.2995	Not significant
All lateral data	0.3043	
All coated tablet data <sup>a</sup> versus	0.2808	Significant at $P = 0.01$
All uncoated tablet data	0.3267	
All large coated tablet data <sup>a</sup> versus	0.2835	Not significant
All small coated tablet data	0.2908	
Coated-heating data	0.3114 ± 0.0052 <sup>b</sup>	
Coated-cooling data	0.2608 ± 0.0053	
Uncoated-heating data	0.3628 ± 0.0070	
Uncoated-cooling data	0.2913 ± 0.0069	

<sup>a</sup> As explained in the text, the data were divided into two groups, the slope of each group calculated, and an analysis of variance made to test the significance of the difference between the slopes. There were four criteria for making the initial division and each was tested.

<sup>b</sup> After determining the significant differences the data were divided into four groups, each already proved different from the other, and the slopes and standard errors were calculated.

TABLE IIA—THE MEAN EXPERIMENTAL  $Z$  VALUES (SPECIFIC HEATING AND COOLING RATES) FOR BOTTLES OF TABLETS

Container, ml	Coated Tablets <sup>a</sup>		Uncoated Tablets <sup>a</sup>	
	$Z_h, \text{min}^{-1}$	$Z_c, \text{min}^{-1}$	$Z_h, \text{min}^{-1}$	$Z_c, \text{min}^{-1}$
28 (7 dr.)	0.088 ± 0.002	0.074 ± 0.002	0.103 ± 0.002	0.083 ± 0.002
50	0.055 ± 0.001	0.046 ± 0.001	0.064 ± 0.001	0.052 ± 0.001
60	0.050 ± 0.001	0.042 ± 0.001	0.058 ± 0.001	0.047 ± 0.001
100	0.041 ± 0.001	0.034 ± 0.001	0.048 ± 0.001	0.039 ± 0.001
120	0.033 ± 0.001	0.027 ± 0.001	0.038 ± 0.001	0.031 ± 0.001
200	0.026 ± 0.0004	0.022 ± 0.0004	0.030 ± 0.001	0.024 ± 0.001
250	0.022 ± 0.0004	0.019 ± 0.0004	0.026 ± 0.001	0.021 ± 0.001

<sup>a</sup> These results are calculated with Eqs. 14-17. Values of  $D$  and  $F_r$ , required for the calculations, are obtained from Table I.

The significantly higher specific rates of heating and cooling of uncoated tablets, as contrasted with coated tablets, is probably due to an actual difference in thermal diffusivity. It is possible that the white uncoated tablets aid radiant heat transfer through the bottle. Heat transfer mechanism is discussed further in a following paragraph.

Table III gives specific heating and cooling rate data for bottles of tablets in cartons. The same heating and cooling processes appear to be followed whether bottles are in or out of cartons. It was noted, however, that the time required to approach an exponential rate of heating and cooling,  $f_h$  and  $f_c$ , was longer in cartons. Also values of  $Z_h$  and  $Z_c$  for bottles in cartons are appreciably smaller than for the same bottles outside of cartons. Position of bottles in cartons also has a bearing on the magnitudes of  $Z_h$  and  $Z_c$ . These magnitudes bear the following interrelationship  $COC > SOC > IOC$ . In the two carton sizes studied,  $Z_h$  and  $Z_c$  values were smaller for the larger carton. This result was expected and is similar to results between different sized bottles alone. No further studies were done with bottled tablets in cartons.

TABLE III.—TABULATED VALUES OF  $Z_h$  AND  $Z_c$  FOR TABLETS IN VARIOUS BOTTLES AND CARTONS

Container	Sample Position	$Z_h$	$Z_c$
50-ml. bottle	Center	0.055	0.046
100-ml. bottle	Center	0.041	0.034
Carton containing 28-ml. bottles <sup>a</sup>	Center, interior of carton	0.016	0.017
	Center, side of carton	0.019	0.020
	Center, corner of carton	0.025	0.026
Carton containing 50-ml. bottles <sup>a</sup>	Center, interior of carton	0.012	0.011
	Center, side of carton	0.015	0.013
	Center, corner of carton	0.019	0.017

<sup>a</sup> In these cases uncoated tablets were used. The values for 50- and 100-ml. bottles were obtained with coated tablets.

**Thermal Diffusivity.**—The approximate linearity of  $Z$  versus  $1/D^2F\pi$  plots permit an estimate of thermal diffusivity to be made. According to Eq. 3, the slope of the straight line portion of these curves equals  $4K$ . Values for  $K$  have been calculated from the slope values given in Table II and are given in Table IV. Literature values of  $K$  for the various materials constituting a bottle of tablets are also included in Table IV. By comparing these data, an estimate may be made of the contribution of the three basic types of heat transfer that are most probable here: solid-solid conduction, gas-solid conduction, and radiation.

The diffusivity of bottled tablets is much too high for heat transfer to be largely dependent upon solid-solid conduction. The increase in  $K$  caused by replacing air in bottles with helium is very small compared to the difference between the  $K$  values of these two gases, and suggests that gas-solid conduction contributes very little to total heat transfer. The major heat transfer thus appears to occur through a radiation mechanism. Convection undoubtedly plays some part in the heat transfer; however, the

TABLE IV.—VALUES OF THERMAL DIFFUSIVITY FOR TABLET FILLED BOTTLES AND TABLET AND BOTTLE MATERIALS

	$K$ , cm. <sup>2</sup> /sec.
Coated tablets in bottles	
Heating	0.078 (0.082) <sup>a</sup>
Cooling	0.065
Uncoated tablets in bottles	
Heating	0.091
Cooling	0.073
Calcium sulfate (CaSO <sub>4</sub> ·2H <sub>2</sub> O)	0.003 <sup>b</sup>
Sugar	0.005 <sup>b</sup>
Glass	0.003 <sup>b</sup>
Air	0.2 <sup>b</sup>
Helium	2.0 <sup>b</sup>

<sup>a</sup> The figure in parenthesis is the  $K$  for a helium-filled bottle full of tablets. The other tablet values are for air-filled bottles. These values are calculated from the slope of the best line for the plot of  $Z = 4K(1/D^2F\pi)$ .

<sup>b</sup> These values are estimates of diffusivity for the various components of bottled tablets. They were calculated from the equation  $K = (\text{thermal conductance/density} \times \text{specific heat})$ . The values for thermal conductance, density, and specific heat were obtained from the "Handbook of Chemistry and Physics," 32nd edition.

tablets offer such a considerable obstacle to gas circulation in the bottle that its contribution to lateral heat transfer must be very small.

**Time for Beginning of Exponential Heating and Cooling.**—As has been noted, extrapolations of the straight line portions of data relating  $\log(T_{ov} - T)$  or  $\log(T - T_r)$  with time, permit estimates of  $f_h$  and  $f_c$ . The latter terms were found to range in magnitude from  $-10$  to  $+15$  minutes, with a mean of  $+3.3$  minutes. For tablets located in the central portions of bottled tablets  $f_h$  and  $f_c$  values increase with increasing bottle size, while for tablets located at the sides of bottles, the  $f$  values have small positive values, or have negative values, indicating that the tablets begin to heat or cool much more rapidly than the centrally located tablets.

From Eq. 13 it can be seen that the difference,  $f_h - f_c$ , rather than the absolute magnitudes of either  $f_h$  or  $f_c$  is important in the calculation of the ETTE value.

Visual inspection of the data for any single bottle indicates that the  $f_h$  and  $f_c$  values for any single tablet of a given bottle were not appreciably different. Statistical evaluation of these data indicated that the mean difference for  $f_c - f_h$  is only  $+0.56$  minutes, with a standard deviation of 1.64. The difference between  $f_c$  and  $f_h$  for any single bottle is so small with respect to the usual ETTE value, in a practical stability study, that the  $f$  terms may be omitted from Eq. 13 without introducing significant error.

**Tablet Temperature Fluctuations.**—One would expect that  $f$ ,  $Z$ ,  $K$ , oven cycle time, and oven temperature variations from the average would be inter-related factors influencing tablet temperature fluctuations during oven storage. For tablets in different positions, bottles, and cartons, where  $f_h$  or  $f_c$  are less than the oven heating cycle, the temperature of a tablet may fluctuate with the same frequency as the oven cycle, but with less variation; however, when the times  $f_h$  or  $f_c$  are more than the oven cycle time, tablet temperature fluctuations would tend to become extremely small. In our studies the oven heating cycle time was 3–7 minutes, with a variation from average of  $\pm 1$  to  $1.5^\circ$ .

Under these heating conditions, no measurable tablet temperature fluctuations were detected. Therefore the study of factors that might influence tablet temperature fluctuations was not pursued further.

**Factors Governing the Effective Tablet Storage Times at Exaggerated Temperatures.**—The errors in kinetic calculations of drug degradation and predictions of shelf life that result from assuming that actual oven storage time and effective storage time are identical are governed by three principal factors: (a) the position of the tablet in the bottle or carton, (b) the approximately exponential heating and cooling processes along with duration of oven storage, (c) the kinetic constants of the breakdown reaction.

It has been noted already that the effects of  $f_A$  and  $f_c$  for any given tablet, may be assumed to cancel each other under conditions of our studies. Therefore, the fact that values may vary from one tablet to another in a given bottle does not introduce error in the determination of storage time under practical exaggerated stability study conditions.

Since the ETTE is independent of  $f_A$  and  $f_c$ , and  $Z_A$  and  $Z_c$  are independent of tablet position, there should be little or no differences in total thermal drug degradation from one tablet to another resulting from differences in tablet position within any single bottle. Likewise, there should be no differences in total thermal drug degradation between tablets in different bottles of similar dimensions which are not in cartons. These deductions are based upon the assumption that the storage oven is operating satisfactorily, is not overloaded, and has a relatively uniform and unobstructed flow of the hot air past each bottle. The previous discussion suggests that if bottle size and fill are kept uniform and the foregoing assumptions met, analytical results of tablet drug content following oven storage should not show significant variation from bottle to bottle nor tablet to tablet within a bottle. We have noted that  $Z_A$  and  $Z_c$  are different for tablets in bottles having different positions within cartons. Taking the IOC position as the reference standard, the error in storage time at other bottle positions, when no correction for position is made, may be calculated from the equation

$$\text{per cent error} = 100 \times$$

$$\frac{\text{ETTE (IOC)} - \text{ETTE (Other Position)}}{\text{ETTE (IOC)}} \quad (\text{Eq. 18})$$

Using Eq. 18, positional storage time errors have been calculated for two different sized cartons for three energies of activation. These are given in Table V from which it may be seen that positional storage time errors increase with carton size. From a practical standpoint, analytical protocols and interpretations of analytical data from exaggerated temperature stability studies would be more straightforward if the bottles were not enclosed in cartons. Therefore it is suggested that such studies never utilize bottles in cartons.

Effective oven storage times are influenced appreciably by the fact that heating and cooling are approximately exponential processes. The magnitude of influence is related to the bottle sizes and actual oven storage times employed. The larger the bottles, the longer the periods for attaining equilibrium oven or room temperatures. The longer the storage

times, the less will be errors resulting from the assumption that the actual storage time is equivalent to the effective storage time at a given temperature. The error that results from the latter assumption may be calculated from Eq. 6

$$\text{per cent storage time error} = 100 \times \frac{t_2 - \text{ETTE}}{\text{ETTE}} \quad (\text{Eq. 6})$$

where  $t_2$  is the actual oven storage time.

One may calculate the effect on the ETTE of the three independent variables,  $t_{or}$ ,  $\Delta H$ , and  $A$ , by utilizing Eqs. 8, 9, and 10. If actual oven time is constant and is of sufficient duration to enable the sample to reach oven temperature, calculations lead to the following conclusions: (a) When  $T_{or}$  is the only variable, there is an increase in the heating time equivalent,  $t_h$ , and a decrease in the cooling time equivalent,  $(t_c - t_2)$  as  $T_{or}$  increases. The ETTE decreases as the difference between  $T_{or}$  and  $T_r$  increases. (b) When  $\Delta H$  is the only variable,  $t_h$  increases and  $t_c$  decreases as  $\Delta H$  increases. The net result is that the ETTE decreases with increasing  $\Delta H$ . (c) The kinetic factor,  $A$ , has no effect on the magnitude of the ETTE, but it has a marked effect upon the magnitude of the specific rate constant for the degradation reaction.

The calculated ETTE values and per cent errors for uncorrected storage times in a hypothetical stability study are given in Table V, for  $\Delta H$  values of 15, 30, and 45 Kcal. Under the experimental conditions of our studies, the errors for uncorrected storage times during, for example, a six-hour period, range from 6–37% for a  $\Delta H$  of 15 Kcal. to 15–157% for a  $\Delta H$  of 45 Kcal. It may be noted that the importance of the storage time error in practical exaggerated temperature stability studies is related to the magnitude of the probability factor  $A$ . For those drugs which possess kinetic constants where, relatively speaking,  $A$  is small and  $\Delta H$  is large, thermal stability will be exceptionally great. Six hours of storage at an exaggerated temperature may result in the thermal degradation of only a fraction of 1% of total drug. Under such conditions a storage time error of  $\pm 2$  hours, for example, would have no important bearing on the determination of the specific velocity constant for thermal degradation,  $k$ , in prediction of shelf life. In such an instance relatively long oven storage times would be required to effect appreciable drug degradation at the elevated temperature. Under the conditions of our experimental studies this would signify that the ETTE values in a kinetic study would approach actual oven storage times, whereupon it becomes unnecessary to make storage time corrections.

Under conditions where  $A$  is relatively large, thermal stability may be very poor. In such an instance, six hours of storage at an exaggerated temperature might result in 10%, or more, thermal degradation. With a drug showing such a characteristic, kinetic determinations of the  $k$  value at this temperature may result in considerable error if the ETTE concepts are not implemented. The ETTE concepts therefore have their greatest utility in calculations pertaining to shelf life prediction or in selecting suitable storage times when dealing with drugs which have marginal thermal stabilities. Thus, when knowledge of drug thermal sensitivity,



TABLE V—ETTE VALUES AND PER CENT STORAGE TIME ERRORS FOR A SIX-HOUR OVEN STORAGE TIME AT 75° CALCULATED FOR VARIOUS BOTTLES, CARTONS, AND ACTIVATION ENERGIES

Container	Position	ETTE <sup>a</sup> min			% Storage Time Error <sup>a</sup> if ETTE Correction Is Not Made		
		$\Delta H =$ 15 Kcal	30 Kcal	45 Kcal	$\Delta H =$ 15 Kcal	30 Kcal	45 Kcal
50 ml	Central	340	321	312	6	12	15
100 ml	Central	334	308	296	8	17	22
250 ml	Central	310	262	240	16	37	50
Carton with 28 ml bottles	IOC	283	223	193	27	61	87
	SOC <sup>b</sup>	296	245	220	22	47	64
	COC <sup>b</sup>	311	272	254	16	32	42
Carton with 50-ml bottles	IOC	264	179	140	37	100	157
	SOC <sup>b</sup>	285	217	181	26	66	99
	COC <sup>b</sup>	300	246	221	20	46	63

<sup>a</sup> Calculations based upon room temperature of 25°<sup>b</sup> The positional storage error for these positions are

		15 Kcal	30 Kcal	45 Kcal
28 ml bottles	SOC	5%	14%	23%
	COC	9%	29%	45%
50 ml bottles	SOC	11%	34%	56%
	COC	17%	54%	94%

acquired prior to formulation, suggests that 10% or more of the drug may degrade at room temperature in less than two years, the application of ETTE concepts in exaggerated temperature studies assumes increasing importance in prediction of product shelf life. When data are available pertaining to the values of  $A$  and  $\Delta H$  for the drug in question in chemical systems other than the proposed formulation, these may serve as a first approximation of the expected  $A$  and  $\Delta H$  values in the tablets or other new formulations of the drug. Computations may then be made to estimate which combinations of storage temperatures, times, and bottles should be selected to program a thermal drug degradation study which will provide convenient confirmatory data upon which to base a shelf life prediction.

The preceding remarks suggest that the practical approach to the simplification of exaggerated temperature stability studies might be to employ actual oven storage times, ( $t_2$ ), where time of heating and cooling to equilibrium temperature is short relative to  $t_2$ . Equation 19 may be obtained algebraically from Eqs 6 and 13 to determine the minimum  $t_2$  which will assure that the actual oven storage time will not differ by more than  $\lambda\%$  from the effective time

$$\text{Min } t_2 = \frac{[Q/Z_h - t_h - (t_c - t_2)][1 + 100/\lambda\%]}{\quad} \quad (\text{Eq 19})$$

Table VI presents figures calculated from Eq 19 which represent minimum oven storage times at 45, 60, and 75° for commercial bottled tablets where, without ETTE correction, storage time errors would be less than 5%. It should be noted that these figures apply to the experimental conditions of this study, and where  $\Delta H$  of the Arrhenius equation equals 15 Kcal. Since  $\Delta H$  will be somewhat variable from one drug to another, it is not possible to establish precise minimum oven storage times, applicable to all tablets, beyond which it is unnecessary to correct for oven storage times. Despite the complications presented by the Arrhenius constants, Table VI is instructive. It suggests that at an oven storage temperature of 75° or lower, actual oven storage times which extend to several days or weeks in bottles of 250 ml or less capacity would

TABLE VI—MINIMUM STORAGE TIMES FOR SEVERAL COMMERCIAL BOTTLES OF TABLETS WHICH INSURE STORAGE TIME ERRORS OF LESS THAN 5%<sup>a</sup>

Bottle ml	$Z_h$ (min <sup>-1</sup> )	Storage Time hr		
		75°C, $Q =$ 4 135	60°C, $Q =$ 3 779	45°C, $Q =$ 3 353
50	0 055	7	3 1/2	Less than
60	0 050	7 3/4	4	1 hour
100	0 041	9 1/2	4 1/2	(see text)
120	0 033	11 1/2	5 1/2	
200	0 026	15	7 1/2	
250	0 022	18	9	

<sup>a</sup> Calculated using the expression  $\text{min } t_2 = 1/60[Q/Z_h - t_h - (t_c - t_2)][(1 + 100/5)]$ . All calculations were done using the hypothetical reaction discussed in Table V where  $\Delta H = 15$  Kcal.

not usually be significantly different from corrected storage times. Also, at the higher storage temperatures, which normally are conducted only for hours or days, one may use the time of Table VI as a standard of reference. Oven storage times at the higher temperatures should be chosen as much in excess of times in Table VI as will permit one to obtain thermal drug degradation data which are amenable to accurate interpretation by chemical kinetic methods. Table VI also suggests that when drug degradation is appreciable after storage for a few hours at elevated oven temperature, advantage in reducing the storage time error is gained by using the smallest bottles and lower storage temperatures. If relatively high storage temperatures seem necessary, an additional step to reduce storage time error might involve the use of Petri dishes, test tubes, or other special containers which facilitate rapid heat transfer.

It may be noted in Table VI that numerical values of minimum  $t_2$  at 45° are not given. This is because they are very small values which would never be employed in a practical oven storage stability study at 45°, however, it is of further interest that the ETTE values for samples stored at this temperature, when calculated according to Eqs 7, 8, and 9, are larger than the actual oven time. This apparently anomalous result occurs because at  $\Delta H =$

15 Kcal., the specific velocity constant,  $k$ , for thermal degradation at 25° is one-fifth that at 45°. This results in a situation where at any time during cooling, the  $k$  of the sample is still an appreciable fraction of the  $k$  at 45°, so that the longer one waits for room temperature equilibration, the more significant becomes the contribution of room temperature breakdown to the ETTE. For the degradation presented, where the  $k$  at  $T$ , is one-fifth that at  $T_{00}$ , each five minutes spent reaching room temperature adds one more minute to the ETTE. When oven temperature increases, this effect diminishes so that it is negligible at 60 and 75°, but is appreciable near 45° and lower temperatures.

**Effect of Product Analysis Time on Shelf Life Prediction.**—The realization that the room temperature degradation rate may not be a negligible factor in the calculation of the ETTE points to another possible source of error in estimating effective storage time in stability studies.

In an industrial pharmaceutical laboratory, analyses are normally performed on a priority basis by analytically trained personnel. Scheduling of analytical work and reporting of analytic data may result in delays between an original product analysis and the time when the product is exposed to exaggerated temperatures for stability study. Likewise there may be a delay between the time the product is removed from a stability study oven and the time of assay. Sometimes these combined time lapses may represent several weeks. That is, a sample stored for a specific time in an exaggerated temperature storage oven may have been stored, in fact, an additional several weeks at room temperature. In those cases where the drugs in question have marginal stabilities, this additional time of room temperature storage may have to be included in the calculations for prediction of shelf life. When, for example, the effective oven storage time at 45° is sixteen days, but the product is maintained an additional fifteen days at room temperature prior to analysis because of programming delays, a better estimate of the effective storage time at 45° might be nineteen days. This estimated time would result if the drug degradation rate at room temperature was, for example, one-fifth of that at 45°.

Though the work reported in this paper has been concerned primarily with tablet heating and cooling characteristics under exaggerated temperature stability study conditions and with the way in which these may affect the accuracy of shelf life predictions based on chemical kinetics, the concepts are also applicable to other dosage forms. Short term exaggerated temperature stability studies are assuming increasing importance in pharmaceutical product development. These studies make it possible to select the most stable product formulas early in the course of a product's development. They also give an indication of the shelf life of a product, and thus furnish an intelligent basis upon which a decision for marketing may be based, or upon which reasonable expiration dates may be selected for products of marginal stability.

Selection of stable formulas early in the course of product development permits the use of the correct product formula in the early stages of clinical tests. This is distinctly advantageous since it gives greater assurance that the final marketed product will per-

form therapeutically in a manner analogous to the results of early clinical evaluation.

The preparation and selection of stable product formulas is normally a development pharmacist's responsibility. The selection of suitably stable formulas and prediction of the product's shelf life based on thermal degradation entails choosing proper exaggerated temperatures, methods, and facilities which assure effective time-temperature storage conditions, packages or containers for the dosage forms, program for withdrawing representative samples for assay, preparation of samples for assay, and finally assay. Despite the frequently high precision of the assay method, the experienced formulator is often faced with assay results of tablets and other dosage forms, which apparently defy the rules of chemical kinetics. Such results may arise, for example, if the original drug isolation and assay method fails to isolate all of the drug from an aged dosage form. Normally such a failure can be easily corrected; however, more subtle reasons for failures that may go undetected result from errors in storage oven heating due to such interrelated factors as, for example, overloading storage ovens, nonuniform temperature within the ovens, improper correlations of stability data derived from products in different sized bottles or cartons, inadequate sampling procedures, or inaccurate estimates of effective storage times and temperatures. Since these factors normally are subject to control by the development pharmacist, it is important that he understands and controls them to the extent that valid thermal degradation data may be obtained when accurate analyses are performed on stability study samples.

More experimental studies which evaluate the importance of each of the numerous factors influencing the collection of thermal drug degradation data would be helpful for improving the procedures employed in drug shelf life prediction.

## SUMMARY

1. The experimental work of this study was concerned with the heating and cooling characteristics of tablets in cylindrical bottles and cartons, when these are placed in hot air ovens. Individual tablet temperatures were measured with a thermocouple and a recorder.

2. The heating and cooling rate coefficients for different tablets in a bottle are relatively independent of their positions in the bottle; however, the apparent times for exponential heating to begin are dependent upon tablet position within a given bottle and the oven storage temperature.

3. Apparent exponential heating or cooling in a given bottle occurs sooner in tablets located at the side of a bottle than for those located centrally.

4. For a given bottle and tablets, the heating and cooling rate coefficients were independent of the oven storage temperatures, i. e., the same values were obtained irrespective of whether oven temperatures were 45, 60, or 75°.

5. Bottles used in these studies ranged in volume from 20 to 250 ml. Standard 12-bottle pasteboard cartons were also used. Times required for bottles and cartons of tablets to attain equilibrium temperatures increased with container size. These times thus increased with increased container heat capacity.

6. For given type tablets, heating and cooling rate coefficients decreased with increasing container size.

7. White uncoated tablets showed heating and cooling rate coefficients about 15 per cent higher than gray-coated tablets of similar size, when studies were done in identical bottles. Large pink sugar-coated tablets showed heating and cooling rate coefficients very similar to smaller gray sugar-coated tablets, when studies were done in identical bottles.

8. Under a given set of experimental conditions, where tablet heating was done in a convected hot air oven and cooling was performed on a laboratory bench, heating rate coefficients were always 15–20 per cent greater than the cooling rate coefficients.

9. Heating and cooling rate coefficients are appreciably smaller for bottled tablets in cartons than for similar bottles outside of cartons.

10. Thermal diffusivity interpretations applied to the data suggest that heat transfer through bottled tablets is mediated largely through a radiation process.

11. Using experimentally determined conductivity values in conjunction with bottle dimensional constants, heating and cooling rate coefficients for coated and uncoated tablets are definable with equations. The equations are presented.

12. The ETTE concept was employed to compute the effective storage time of tablets stored under different experimental conditions and these values were compared with actual oven storage times. For example, a 250-ml. bottle filled with large coated tablets and stored for two hundred and forty minutes at 75° has an effective storage time of one hundred and ninety minutes when  $\Delta H = 15$  Kcal., and a first-order rate of degradation is assumed.

13. The effects that variables  $A$  (probability factor),  $\Delta H$ , oven temperature, and bottle size have on the computed value of ETTE for a drug in tablets are indicated. Also indicated is how these variables relate to the programming of exaggerated temperature stability studies.

14. A table has been presented giving minimal storage times of different sized bottled tablets at several oven temperatures which can serve

as a guide for programming exaggerated temperature stability studies. Use of the table can serve as a guide to the selection of oven temperatures, bottle sizes, and storage times which circumvent the need of ETTE calculations of oven storage time, thereby simplifying calculation of reasonable estimates of product shelf life.

15. It has been noted that the validity of exaggerated temperature stability data may be affected adversely by failure to correct for finite heating and cooling times of samples; also by other interrelated factors such as overloading storage ovens, nonuniform temperature within the ovens, improper correlations of stability data derived from products in different sized containers, and inadequate sampling procedures. Since such factors normally can be controlled by the development pharmacist, it is desirable that he understands the degree of their importance in specific stability studies; also that he exercises the measure of control necessary to give confidence to calculations of product shelf life at room temperature.

## GLOSSARY OF TERMS

All temperatures in degrees Kelvin,<sup>4</sup> times in minutes, and dimensions in centimeters.

$T_{ov}$	= oven temperature
$T$	= sample tablet temperature
$T_r$	= room temperature
$T_{95\%}$	= temperature at which the specific breakdown rate equals $0.95 k_{01}$
$t$	= time
$t_0$	= time when sample is placed in the oven ( $t = 0$ )
$t_1$	= time after $t_0$ for sample to reach oven temperature
$t_2$	= time after $t_0$ when sample is removed from oven
$t_3$	= time after $t_0$ for sample to reach room temperature again
$t_h$	= time after $t_0$ for sample at $T_{ov}$ to break down an amount equal to that occurring in a heating sample during its heating cycle (the heating time equivalent)
$(t_c - t_2)$	= time for a sample at $T_{ov}$ to break down an amount equal to that occurring in a cooling sample during its cooling cycle (the cooling time equivalent)
$Z_h$	= specific heating rate (min. <sup>-1</sup> )
$Z_c$	= specific cooling rate (min. <sup>-1</sup> )
$f_h$	= time difference between $t_0$ and the apparent beginning of logarithmic heating

<sup>4</sup> When temperature differences are measured, i. e.,  $T_{ov} - T_r$ , degrees centigrade may be used, giving values identical to those that result when  $T$  values are expressed in degrees Kelvin.

$t_c$	= time difference between $t_2$ and the apparent beginning of logarithmic cooling
$D$	= diameter of bottle studied
$H$	= height of bottle studied
$K$	= diffusivity constant ( $\text{cm}^2/\text{sec}$ )
$A$	= kinetic probability constant ( $10^5 \text{ min.}^{-1}$ )
$\Delta H$	= heat of activation of the breakdown reaction (15, 30, or 45 Kcal /mole)
$R$	= gas constant ( $1.987 \text{ cal deg}^{-1} \text{ mole}^{-1}$ )
$k_{at}$	= specific reaction rate at oven temperature ( $\text{min.}^{-1}$ )
ETTE	= equilibrium temperature time equivalent (units of time)

$Q$	= $(2.303 \{ \log (T_{ov} - T_r) - \log (T_{ov} - T_{95\%}) \})$ (dimensionless)
$P_h$	= numerical solution of the integral of Eq 8 (dimensionless)
$P_c$	= numerical solution of the integral of Eq 9 (dimensionless)

## REFERENCES

- (1) Eriksen, S. P., Pauls, J. F., and Swintosky, J. V., *THIS JOURNAL*, 47, 697(1958)
- (2) Ball, C. O., *Natl. Research Council Bull.*, 1923, 37
- (3) Ball, C. O., *Univ. Calif. Berkeley Pubs. Public Health*, 1, 15(1928)
- (4) Stumbo, C. R., *Advances in Food Research*, 2, 47(1949)
- (5) Williamson, E. D., and Adams, L. H., *Physiol. Revs.* 14, 99(1919)

## Some Effects of Ionized Air on *Penicillium notatum*\*

By ROBERTSON PRATT and ROBERT W. BARNARD

Exposure of *Penicillium notatum* to ionized air reduces the production of penicillin, negative ions exerting a greater effect than positive ions. Growth of the mycelium and germination of spores also are depressed, but positive ions exert a greater effect than negative ions on spore germination. Production of carbon dioxide is depressed to about the same extent as mycelial growth.

ABOUT two hundred years ago, the dependence of various biologic functions on atmospheric electricity, both natural and artificially produced, was investigated, and many experiments purporting to show effects upon secretions, blood, pulse, growth, mental activity, and even morality, were described. Disruptive discharge was used therapeutically in Halle, Germany, in 1744 to influence the course of disease, and following description of a method in 1754 to produce positive and negative air ions, aniontherapy was introduced. Sporadic accounts of biologic and therapeutic effects of ionized air have continued to appear through the intervening years, including several reports that an excess of negative ions existing in the atmosphere at some of the most famous spas of Europe is partly responsible for the supposed health-restoring qualities of those resorts. The earlier literature, much of which is in the category of testimonials and speculation, has been reviewed (1). In our own time, nega-

tive air ions have been reliably reported to relieve and positive ions to aggravate sinusitis, rhinitis, asthma, and pollenosis in humans (2, 3), and positive air ionization has been reputed to induce headache, dizziness, fatigue, and malaise, while negative air ions have been said to ease the breathing and induce a sense of well being (4). These reports deal with subjective clinical responses.

Objective laboratory research has revealed significant quantitatively measurable effects of ionized air on various biologic systems. For example, exposure to positively ionized air causes a reduction in the succinoxidase content of the adrenal gland of the intact rat (5), both positive and negative air ions are lethal to staphylococci (6), and negative air ions increase and positive air ions decrease or abolish ciliary movement in mammalian trachea, both *in vivo* and *in vitro* (7-9).

This report is concerned with effects of air ionization on certain activities of the fungus, *Penicillium notatum*. Antibiotic production by the mold was chosen as the major activity for study because it is an easily and reproducibly measurable objective index of biochemical activity. The effects of air ionization on spore germination and mycelial growth of the mold and on its carbon dioxide production also were observed.

\* Received December 5, 1959 from the School of Pharmacy, University of California Medical Center, San Francisco 22.

## AIR IONIZATION

Ionization differs in gaseous and liquid media. Ionization in a liquid results from separation of molecules into anions and cations, whereas in a gaseous medium it results from removal of an electron or substitution with a particle of different electrical charge (10). The ions may be small, consisting of about a dozen gaseous molecules associated with a single electric charge; intermediate, consisting of about 100 submicroscopic molecules bearing a charge; or large, i. e., about 1,000 times the size of small ions (11). The normal atmosphere contains from 500 to 2,000 small and intermediate positive air ions per  $\text{cm}^3$  and a slightly smaller number of negative air ions (12). The ions range from 0.001 to 0.1  $\mu$  in diameter. The chief natural sources of air ionization are cosmic rays and radioactive particles in the air and earth. Such particles are derived mainly from the disintegration of thorium and radium which emit alpha, beta, and gamma radiation.

Air ions may be generated artificially by thermionic methods, or by the use of radioactive materials, X-rays, photoionization, high voltage brush discharges, high frequency currents, and atomization of water, the latter accounting for the high density of negative air ionization in the immediate vicinity of water falls and some spas. These methods have been reviewed (13-15). Accurate measurement of atmospheric electrostatic potential can be made with an ion current probe (16), and effective climate control through regulation of air ionization has been reported (14, 17).

**Ion Generator.**—The ion generators employed in the present work have a radioactive tritium-titanium foil as a source of beta radiation.<sup>1</sup> The beta rays collide with air molecules and particles and create both positive and negative ions. By means of a rectifier, a unipolar charge is established on a metal plate near the radioactive foil. The plate attracts and traps ions with an opposite charge and repels into the atmosphere ions with a like charge. The velocity of the air ions depends on their size and on the voltage on the rectifier plate. Velocities of the biologically important "small"- and "intermediate"-sized ions vary from about 0.01 to about 22 cm./sec./volt/cm. (18). To generate an atmosphere rich in negative ions, the plate is charged negatively. Reversing the polarity will produce a high density of positive ions. Many of the details and much of the theory have been reviewed (15, 19-21).

The foils in the ion generators have an area of about 0.4  $\text{cm}^2$ , and each one contains about 50 mc. of tritium. Tritium is a beta ray emitter with an energy of irradiation of 0.015 Mev. The approximate range of radiation at this intensity is 0.002 Gm./ $\text{cm}^2$  (19). It has been calculated that in air (density 1.2 mg./ml.) the maximum range is 1.7 cm. (6). Since the units were so placed that the target surfaces (*Penicillium* cultures) were 5 cm. distant, the possibility of direct radiation effects on the organism was minimized. Nevertheless, special radiation controls were run in each experiment. At the rectifier voltage employed (860 v.), each unit de-

livered  $10^6$  ions per  $\text{mm}^2$  of culture surface per second. Cultures were exposed continuously throughout each experiment.

## EXPERIMENTS AND RESULTS

**Antibiotic Biosynthesis.**—*Penicillium notatum* ATCC 9178 (NRRL 1249.B21), a surface culture strain of the mold, was used throughout. Technical details of the design of the ionizer equipment made it impractical to study the effect of ionized air on submerged cultures; therefore, surface culture methods were employed. This was considered justifiable, despite the fact that industrial production is by means of submerged culture techniques, since the experiments were aimed at determining whether or not air ions influence biochemical reactions of the mold and not at industrial application. Moreover, because of the low penetrability and the rapid neutralization and dissipation of air ions in aqueous media, it is doubtful that any effect could be observed in submerged cultures even if the available equipment had been adaptable to such an experimental set-up.

The culture medium had the following composition:  $\text{KH}_2\text{PO}_4$ , 0.057 M;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.006 M;  $\text{NaNO}_3$ , 0.057 M; phenylacetic acid, 0.00183 M;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.00015 M; lactose, 0.111 M; and corn steep liquor solids 2%.

The geometry of the ionizing units limited the choice of glassware for culture vessels. Berzelius beakers (100-ml. size) containing 50 ml. of the above medium plus 2 ml. of inoculum finally were selected. The area of the air-surface interface was 18.1  $\text{cm}^2$  and, consequently, the ratio of surface to volume approached 1:3. The yields of penicillin recorded below for control cultures are considerably less than those the organism is capable of producing, but they are not excessively low in view of the relatively unfavorable surface/volume ratio and in view of the light inoculum that was used in order to avoid excessive vegetative growth which would have shielded underlying mycelia from the air ions.

Antibiotic activity was determined by the standard cylinder plate technique, using *Micrococcus pyogenes* var. *aureus* ATCC 6538 P (*Staphylococcus aureus* FDA 209 P), as the test organism.

Data from preliminary experiments indicated that air ionization exerts an effect on the yield of penicillin and that the magnitude of the effect depends on the density of the seeding inoculum and on the duration of the experiment. For example, after seven days of incubation, the yield of penicillin in cultures exposed to negative air ions was reduced about 80% in comparison with nonionized controls when a very sparse inoculum was used, and about 22% when a very dense inoculum was used. The procedure adopted was standardized by using an inoculum of intermediate density which resulted in approximately 50% reduction in yield after seven days. The inoculum consisted of 2 ml. of a spore suspension per 50 ml. of culture medium. The suspension was prepared from three-day-old cultures and was adjusted to 10% transmission as determined by a Lumetron, model 402 EF equipped with a neutral filter.

As a precaution, two controls were run for each experiment, one with no ionizing unit and one a

<sup>1</sup> The ion generators were kindly furnished by Dr. J. C. Beckett of the Wesix Electric Heater Co., San Francisco, Calif.

radiation control to check the effect, if any, of secondary beta radiation. This control consisted of a culture exposed to an ionizing unit which was not connected to the power line. Thus, this culture was exposed to the same radiation as the experimental cultures, but was not exposed to a differential air ionic atmosphere. The ambient atmosphere in this culture vessel was richer in air ions of both polarities, due to the ionizing action of the beta rays. However, in the small confined volume over the cultures, the negative ions could be expected to combine with positive ones, thus tending to restore polar neutrality. That this occurred, and that the results observed in the experimental cultures were due to the air ions and not to a radiation effect *per se*, was indicated by the fact that in all experiments the radiation controls and the standard controls gave identical values within the experimental limits stated in Table I. All experiments were performed in a constant temperature room maintained between 24 and 26°.

Data from five replicated experiments summarized in Table I show that both negative and positive ionization of the air reduced the yield of penicillin, and that negative ions had a more depressing effect than positive ions.

TABLE I.—EFFECT OF NEGATIVE AND POSITIVE AIR IONS ON YIELD OF PENICILLIN AND ON DRY WEIGHT OF MYCELIUM IN CULTURES OF *Penicillium notatum*

Ionization	Penicillin, units/ml. <sup>a</sup>		Dry Weight of Mycelium, mg. <sup>b</sup>
	7 Days	10 Days	10 Days
None (control)	73	86	771.1
Negative	38.6	53.9	616.9
Positive	55.8	78	674.7

<sup>a</sup> Maximum deviation among quadruplicate assays in any one experiment  $\pm 3\%$ , and among the several experiments  $\pm 5\%$ .

<sup>b</sup> Maximum deviation from the average among the several experiments  $\pm 7.2\%$ .

It seemed possible, although only remotely so, that the effect on yield of penicillin was not primarily a biological one exerted through the mold, but instead was primarily a physical or chemical one involving destruction of the antibiotic after it was biosynthesized, or perhaps was a combination of the two effects. For example, the reduction in yield of antibiotic activity in cultures exposed to negative ions was considerably greater than might have been expected from the decrease in growth, although it is well known that there is not necessarily a concordance between vegetative growth and antibiotic production in cultures of *Penicillium*. The possibility seemed to exist that equal or nearly equal amounts of antibiotic were produced in all cultures but that the rate of destruction was greater in an atmosphere with a high density of negative ions. Accordingly, sterile filtrate from a seven-day *Penicillium* culture was aseptically apportioned into four sterile culture vessels, one of which was supplied with positively and one with negatively ionized air while the other two served as the usual controls. All were placed in the incubator room. Aliquots were removed aseptically from each vessel daily for seven days and were assayed. The loss in potency

was the same in all vessels. Therefore, it seems safe to assume that the ionized air did not have any direct effect on the antibiotic liberated into the culture medium but that the effect was exerted on the fungus.

Comparison of the data for seven days and ten days in Table I suggests that the effect of ionization on yield of antibiotic decreased with time. The penetrating ability of air ions is low. Consequently, only the cells in the mycelium comprising the uppermost surface of the mold mat were exposed, and, therefore, as the thickness of the mold mat increased, an increasing proportion of the mycelial cells was protected from the ionized atmosphere and its detrimental effect on antibiotic synthesis. By the tenth day there was no significant difference in yield from control cultures and those exposed to positive air ions, although the negative ion cultures still showed appreciable depression.

**Spore Germination and Mycelial Growth.**—Microcultures, consisting of *Penicillium* spores from three-day-old sporulation cultures seeded onto a thin layer of sporulation agar on cover glasses maintained in a humid atmosphere, were exposed to the ionizing units. Five to ten microscope fields, containing 250 to 300 spores, on each slide were examined periodically. There was considerable delay in germination of spores exposed to ionized air of either polarity. For example, at the time (seven hours) when 4% of control spores had germinated, less than 1% germination had occurred in cultures exposed to either negative or positive ionization. After nine hours, 63% of the control spores, 40.7% of the "negative" spores, and 30% of the "positive" spores had germinated. At that time, average germ tube length was 5.2  $\mu$  for the control and 4.4  $\mu$  for each of the experimental cultures. Observations were not continued beyond nine hours because after that time the growth pattern of the germ tubes made accurate observation and measurement impossible.

The observations on spore germination are consistent with similar studies made on *Neurospora crassa*, in which ionization of either polarity markedly reduced spore germination and positive ionization was found to be more detrimental than negative ionization (22).

The data in Table I show that growth of the mold (as indicated by dry weight of mycelium formed) was reduced approximately 12 to 20% when cultures were exposed to an atmosphere artificially enriched in air ions for ten days.

**Carbon Dioxide Production.**—Pyrex tubes were annealed to opposite sides of the culture vessels and the vessels were made part of a gas train, being both preceded and followed by potassium hydroxide traps. By applying gentle suction at one end of the train, carbon dioxide-free air was drawn into the culture chamber at a rate of 50 ml. per minute and the carbon dioxide evolved by the cultures was trapped in the succeeding hydroxide tubes. At the end of the experiment, the base in these tubes was titrated against standard HCl to the phenolphthalein end point. Barium chloride was added to remove the soluble carbonate that could act as a buffer. At the end of the run, the mold mats were carefully removed from the culture vessels and dried to constant weight. The titration data were converted to carbon dioxide output per 100 mg. of mycelium.

Values for carbon dioxide produced by control, by "positive," and by "negative" cultures were 159.5 ml, 147.2 ml, and 134.3 ml, respectively, per 100 mg dry weight of mold over a five-day period. Thus, taking carbon dioxide production of the control cultures as 100%, the corresponding figures for "positive" and "negative" cultures were 92.3% and 84.2%, respectively.

## DISCUSSION

It has been shown that spore germination and growth of *Penicillium notatum* and biosynthesis of penicillin are reduced when the organism is exposed to an atmosphere artificially enriched in either negative or positive air ions, but that the effects of the two polarities are not identical and the functions measured are not affected to the same degree.

Whereas negative ionization reduced growth about 20% over a ten day period, it reduced antibiotic production almost 40% in the same period of time. Such a large difference cannot be ignored. It suggests involvement of at least two different enzyme systems, one of which is more subject than the other to the influence of the ions. It has been established that enzymes can be influenced by air ions. For example, Nielson and Harper (5) showed that when intact rats were maintained in an atmosphere of positively charged air ions for only four hours, the succinoxidase content of the adrenal gland was diminished 13% as compared with control animals. In the present experiments, the mold was exposed continuously for periods up to ten days. Recent work of other investigators suggests that an important target enzyme of air ions is the cytochrome oxidase system (23).

The action of air ionization may affect enzyme systems to produce a molecule with qualitatively (or quantitatively) different antibiotic activity than is normally formed. If this were happening it would not have been detected in the present experiments because assays were performed with only one test organism. This problem should be pursued by the use of chromatographic or other suitable techniques and by bioassays employing multiple test organisms. This is of some interest, since the data on carbon dioxide production, corrected for weight of the mold mat in the different cultures, indicate that respiration proceeds at a lower rate in cultures exposed to ionized air than in the controls. The possibility that air ions alter the permeability of the mycelial cells should not be ignored. Conceivably, nutrients might not be so readily absorbed, thereby, in effect, giving the organism less raw material with which to work or, alternatively, intermediates in the biosynthetic reactions may leach out of the cells before the final antibiotic molecule is formed. In either case lower yields of penicillin might be expected. Further experimentation is needed to evaluate these and other possibilities.

## SUMMARY

The literature on air ionization has been reviewed briefly, with particular reference to its

biological effects. Although such effects have been reported sporadically for more than two hundred years, many of the reports must be classified as subjective. In recent years the number of reports of objectively demonstrable effects of air ionization on living cells, tissues, and organs has been increasing.

The experiments reported in the present paper showed that air ions of either polarity have a pronounced effect on the ability of *Penicillium notatum* to produce penicillin in surface culture, on the ability of the fungus spores to germinate, and on the vegetative growth of the mold. Behavior of the organism in normal air, i.e., air not carrying a heavier load of one ionic form than the other, was used as a reference for comparison. An atmosphere rich in negative air ions had a greater depressing effect than one rich in positive ions on the synthesis of antibiotic, but, with respect to spore germination, the positive ions were more deleterious than the negative ones.

Preliminary experiments have suggested that carbon dioxide production, corrected for differences in mycelial weight, also is suppressed by both positive and negative air ions and that the negative ions produce a greater depressing effect than the positive ions.

## REFERENCES

- (1) Edstrom, G. *Acta Med Scand Suppl*, 61, 1(1935)
- (2) Kornbluh, I. H., and Griffin, J. E., *Am J Phys Med*, 34, 618(1955)
- (3) Kornbluh, I. H., et al., *ibid*, 37, 18(1958)
- (4) Winsor, T., and Beckett, J. C., *ibid*, 37, 83(1958)
- (5) Nielson, C. B., and Harper, H. A., *Proc Soc Exptl Biol Med*, 86, 753(1954)
- (6) Kreuger, A. P., et al., *J Gen Physiol*, 41, 359(1957)
- (7) Kreuger, A. P., and Smith, R. F., *Proc Soc Exptl Biol Med*, 96, 807(1957)
- (8) Kreuger, A. P., and Smith, R. F., *ibid*, 98, 412(1958)
- (9) Kreuger, A. P., and Smith, R. F., *J Gen Physiol*, 42, 69(1958)
- (10) Windschbaur, A., "Die Natürlichen Heilkräfte von Bad Gastein," Springer Verlag, Wien, 1948
- (11) Dessauer, F., "Zehn Jahre Forschung auf dem Physikalisch Medizinischen Grenzgebiet," Georg Thieme, Leipzig, Germany, 1931
- (12) Chalmers, A., "Atmospheric Electricity," Clarendon Press, Oxford, England, 1949
- (13) Crowther, J. A., "Ions, Electrons and Ionizing Radiations," 8th ed., Longmans, Green and Co., New York, N. Y., 1949
- (14) Martin, T. L., Jr., *J Franklin Inst*, 254, 4(1952)
- (15) Martin, T. L., Jr., *Trans Am Inst Elec Engrs, Part I*, 72, 771(1954)
- (16) Kreuger, A. P., et al., *J Franklin Inst*, 266, 9(1958)
- (17) Skilling, H. H., and Beckett, J. C., *ibid*, 256, 5(1953)
- (18) Hicks, W. W., and Beckett, J. C., "The Control of Air Ionization and Its Biologic Effects," paper presented at meeting of International Society for the Study of Bioclimatology, Paris, France, August 28-31, 1956
- (19) Glasser, O., "Medical Physics," Yearbook Publishers Chicago, 1944, pp. 644, 715
- (20) Massey, H. S. W., "Negative Ions," University Press Cambridge, England, 1950
- (21) Pollard, E. C., *Soc American*, 191, 63(1954)
- (22) Fuert, R., Annual Report M. D. Anderson Hospital and Tumor Institute, University of Texas, 1955, p. 60
- (23) Kreuger, A. P., and Smith, R. F., Proceedings First International Symposium on Submarine and Space Medicine, Sept. 8-12, 1958, New London, Conn.

# Rat Bioassay of Combinations of Diuretics\*

By MARIO D. G. ACETO† and CASIMIR T. ICHNIOWSKI

A bioassay procedure was used to determine whether or not combinations of acetazolamide sodium with selected diuretic agents would yield more effective responses than those induced by the individual components of a given combination. The diuretics chosen for combination with acetazolamide sodium were: caffeine, mercuraphylline, potassium acetate, potassium bicarbonate, potassium citrate, potassium nitrate, sodium acetate, theobromine, and theophylline. The best combinations of relatively weak diuretics were obtained with the acetazolamide sodium + potassium bicarbonate and acetazolamide sodium + potassium acetate series. Both the acetazolamide sodium + caffeine and acetazolamide sodium + potassium nitrate combinations warrant more critical evaluation.

THIS STUDY was undertaken to determine whether or not combinations of acetazolamide sodium<sup>1</sup> with selected diuretics would yield responses better than those induced by the individual components of a given combination. It was also desired to determine if weak doses of two diuretic compounds could be combined and provide a diuresis equal to or greater than that elicited by a larger dose of each component of a combination. If effective responses could be obtained with these combinations such mixtures might offer the advantage of decreased incidence of side effects without loss of desired pharmacologic response.

## EXPERIMENTAL

**Materials.**—The acetazolamide sodium used in these experiments was furnished by Lederle Laboratories.<sup>2</sup> Other diuretics employed were the official products: caffeine, mercuraphylline, potassium acetate, potassium bicarbonate, potassium citrate, potassium nitrate, sodium acetate, theobromine, and theophylline. Acaia U S P was used as the suspending agent for the poorly water-soluble caffeine, theobromine, and theophylline.

**Procedure.**—The general procedure for rat diuretic tests described by Lipschitz, *et al.* (1), was followed. In this study, Wistar strain rats of both sexes in the weight range 110 to 400 Gm. were used. Urine samples were collected in graduated glass cylinders over which were superimposed metal metabolism cages housing the medicated animals. Combinations of two components only were studied. The constant member of each binary mixture was acetazolamide sodium. A 4% acaia mucilage in normal saline was used to prepare stock suspensions of the xanthines. These stock preparations, diluted with an equal volume of saline, were thoroughly shaken just prior to administration. All other solutions were prepared with normal saline as solvent.

\* Received November 2, 1959, from the School of Pharmacy, University of Maryland, Baltimore.

† Abstracted in part from a thesis submitted by Mario D. G. Aceto to the Graduate School of the University of Maryland, Master of Science.

University of Pittsburgh, Pittsburgh, Pa.

<sup>1</sup> Diamox Sodium is the registered trade name of Lederle Laboratories for acetazolamide sodium.

<sup>2</sup> The authors express their gratitude to Dr. Christopher H. Demos of the Lederle Laboratories for the generous supply of this material.

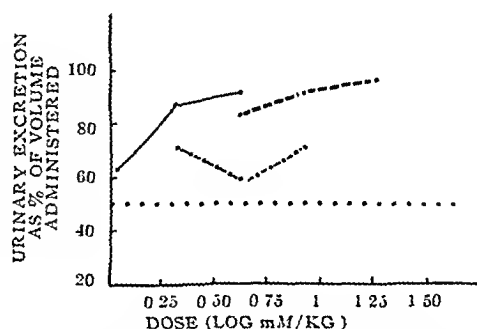


Fig. 1.—Results of acetazolamide sodium + potassium acetate assay A: . . . ., control; ----, acetazolamide sodium (dose  $\times 10^3$ ); - · - · - ·, potassium acetate; —, acetazolamide sodium + potassium acetate [doses in the combined curve expressed as mM/Kg. are: (a) acetazolamide sodium 0.002 + potassium acetate 1, (b) acetazolamide sodium 0.002 + potassium acetate 2, (c) acetazolamide sodium 0.002 + potassium acetate 4]. All doses in figure are expressed as log mM/Kg.

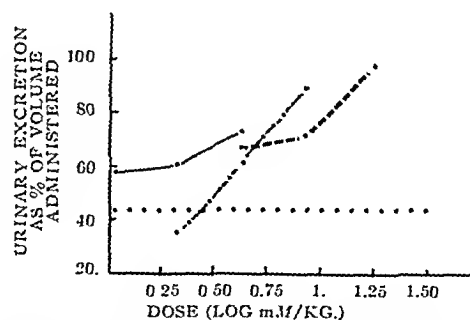


Fig. 2.—Results of acetazolamide sodium + potassium acetate assay B: . . . ., control; ----, acetazolamide sodium (dose  $\times 10^3$ ); - · - · - ·, potassium acetate; —, acetazolamide sodium + potassium acetate [doses in the combined curve expressed as mM/Kg. are: (a) acetazolamide sodium 0.002 + potassium acetate 1, (b) acetazolamide sodium 0.002 + potassium acetate 2, (c) acetazolamide sodium 0.002 + potassium acetate 4]. All doses in figure are expressed as log mM/Kg.

Eight rats were used for each dose level tested. In any given series of tests, the experimental groups were of approximately equal weight. All administrations were made intragastrically. The



TABLE I.—RESULTS OF TWO ASSAYS: ACETAZOLAMIDE SODIUM-POTASSIUM BICARBONATE SERIES

Materials Administered	Dose, mM/Kg.	Urinary Excretion as Per Cent of Volume Administered	
		Assay A	Assay B
Saline	...	69.2	39.5
Acetazolamide sodium	0.002	62.4	...
Acetazolamide sodium	0.004	59.5	60.5
Acetazolamide sodium	0.008	87.7	67
Acetazolamide sodium	0.017	...	66.4
Potassium bicarbonate	1.1	44.4	...
Potassium bicarbonate	2.2	51.8	...
Potassium bicarbonate	4.4	61.9	...
Potassium bicarbonate	3	...	48.4
Potassium bicarbonate	6	...	62.5
Potassium bicarbonate	12	...	96.8
Acetazolamide sodium	0.002	...	...
+	+	65	...
Potassium bicarbonate	1.1	...	...
Acetazolamide sodium	0.004	...	...
+	+	72.4	..
Potassium bicarbonate	2.2	...	...
Acetazolamide sodium	0.008	...	...
+	+	103.1	87.1
Potassium bicarbonate	4.4	...	...
Acetazolamide sodium	0.004	...	96.1
+	+	...	...
Potassium bicarbonate	4.4	...	...
Acetazolamide sodium	0.017	...	100.8
+	+	...	...
Potassium bicarbonate	4.4	...	...

TABLE II.—RESULTS OF THREE ASSAYS: ACETAZOLAMIDE SODIUM-POTASSIUM NITRATE SERIES

Materials Administered	Dose, mM/Kg.	Urinary Excretion as Per Cent of Volume Administered		
		Assay A	Assay B	Assay C
Saline	...	38.8	44.8	46.1
Acetazolamide sodium	0.004	46.5	43.2	64.8
Acetazolamide sodium	0.008	66	59.2	74
Acetazolamide sodium	0.017	86.3	68.6	91.1
Potassium nitrate	1.25	45.4	25.4	38.3
Potassium nitrate	2.5	58.9	42.9	59.6
Potassium nitrate	5	93.1	83.1	81.2
Acetazolamide sodium	0.002	...	...	...
+	+	68.1	58.6	57.7
Potassium nitrate	0.62	...	...	...
Acetazolamide sodium	0.004	...	...	...
+	+	94.5	...	...
Potassium nitrate	1.25	...	...	...
Acetazolamide sodium	0.008	...	...	...
+	+	109.7	...	...
Potassium nitrate	2.5	...	...	...
Acetazolamide sodium	0.002	...	42.1	52.3
+	+	...	...	...
Potassium nitrate	1.25	...	...	...
Acetazolamide sodium	0.002	...	65.3	63.2
+	+	...	...	...
Potassium nitrate	2.5	...	...	...

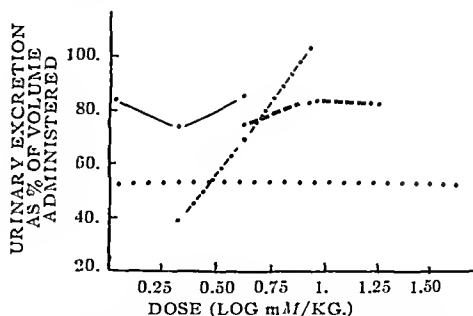
Fig. 3.—Results of acetazolamide sodium + potassium acetate assay C: ..... , control; ----, acetazolamide sodium (dose  $\times 10^3$ ); - · - · - , potassium acetate; —, acetazolamide sodium + potassium acetate [doses in the combined curve expressed as mM/Kg. are: (a) acetazolamide sodium 0.002 + potassium acetate 1, (b) acetazolamide sodium 0.002 + potassium acetate 2, (c) acetazolamide sodium 0.002 + potassium acetate 4]. All doses in figure are expressed as log mM/Kg.

TABLE III.—RESULTS OF THREE ASSAYS: ACETAZOLAMIDE SODIUM-CAFFEINE SERIES

Materials Administered	Dose, mM/Kg.	Urinary Excretion as Per Cent of Volume Administered		
		Assay A	Assay B	Assay C
Saline	...	40.8	43.5	55.2
Acacia in saline	...	...	...	56.9
Acetazolamide sodium	0.004	43.5	80.8	80.2
Acetazolamide sodium	0.008	56.5	67.6	66.3
Acetazolamide sodium	0.017	61.2	85.6	100
Acetazolamide sodium in acacia-saline	0.004	...	...	62
Acetazolamide sodium in acacia-saline	0.008	...	...	82.8
Acetazolamide sodium in acacia-saline	0.017	...	...	87.6
Caffeine	0.1	55.9	70.5	74.7
Caffeine	0.2	44.1	52.5	79.9
Caffeine	0.4	61.2	76.9	73.9
Acetazolamide sodium	0.008	...	...	...
+	+	91.1	...	...
Caffeine	0.1	...	...	...
Acetazolamide sodium	0.008	...	...	...
+	+	87.6	...	...
Caffeine	0.2	...	...	...
Acetazolamide sodium	0.008	...	...	...
+	+	107.7	...	...
Caffeine	0.4	...	...	...
Acetazolamide sodium	0.002	...	48	...
+	+	...	...	...
Caffeine	0.1	...	...	...
Acetazolamide sodium	0.002	...	77.1	...
+	+	...	...	...
Caffeine	0.2	...	...	...
Acetazolamide sodium	0.002	...	...	...
+	+	...	104.9	117.7
Caffeine	0.4	...	...	...

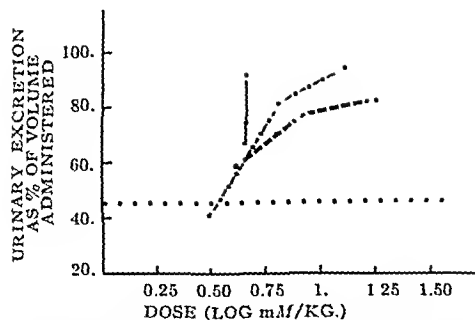


Fig. 4.—Results of acetazolamide sodium + potassium bicarbonate assay C: ..... , control; -----, acetazolamide sodium (dose  $\times 10^3$ ); - · - · - ·, potassium bicarbonate; ———, acetazolamide sodium + potassium bicarbonate [doses in the combined curve expressed as mM/Kg. are: (a) acetazolamide sodium 0.002 + potassium bicarbonate 4.4, (b) acetazolamide sodium 0.004 + potassium bicarbonate 4.4, (c) acetazolamide sodium 0.008 + potassium bicarbonate 4.4]. All doses in figure are expressed as log mM/Kg.

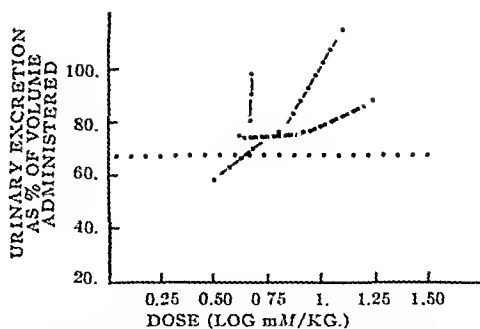


Fig. 5.—Results of acetazolamide sodium + potassium bicarbonate assay D: ..... , control; -----, acetazolamide sodium (dose  $\times 10^3$ ); - · - · - ·, potassium bicarbonate; ———, acetazolamide sodium + potassium bicarbonate [doses in the combined curve expressed as mM/Kg. are: (a) acetazolamide sodium 0.002 + potassium bicarbonate 4.4, (b) acetazolamide sodium 0.004 + potassium bicarbonate 4.4, (c) acetazolamide sodium 0.008 + potassium bicarbonate 4.4]. All doses in figure are expressed as log mM/Kg.

volume administered to each animal was 25 cc./Kg. Dose-effect relationships were established for each diuretic material. Based on these data, combinations of acetazolamide sodium with other individual diuretics were prepared and screened for effect. The promising combinations were then subjected to further study. An estimate of the activity of a diuretic combination included, on any given day of the test, a comparison of the dose-response relationships of the combinations with those of the individual components of the combination.

## RESULTS AND DISCUSSION

The two series considered the most effective combinations of weak diuretic doses were acetazolamide sodium with potassium bicarbonate and the carbonic anhydrase inhibitor with potassium acetate. The various interrelationships in these two series are given in Figs. 1 to 5. The slopes representing the acetazolamide sodium + potassium bicarbonate dose-effect relationships in Figs. 4 and 5 appear perpendicular because, for each dose combination, the dose for potassium bicarbonate remained constant at 4.4 mM/Kg. and the dose for acetazolamide sodium varied only from 0.002 to 0.008 mM/Kg. Table I presents additional similar data obtained

from other assays in the acetazolamide sodium + potassium bicarbonate series not presented in graph form.

Although certain dose combinations in both the acetazolamide sodium + potassium nitrate and acetazolamide sodium + caffeine series indicated merit in combining weak doses of the components, as shown in Tables II and III, respectively, it is felt that the limited testing precludes a general conclusion.

Tests carried out with potassium citrate, sodium acetate, theophylline, and theobromine were indicative that these diuretics offered little promise when combined with the carbonic anhydrase inhibitor.

Attempts to include mercuraphylline in this study were unsuccessful. Inconsistent results were obtained with doses ranging from 0.01 to 81.0 mg./Kg. Even when the assay time was extended to eight hours to determine any delayed action of the mercurial, no increase in diuretic effect occurred.

With regard to the xanthine diuretics, the inconsistencies and difficulties encountered by Lipschitz, *et al.* (1), in the main were confirmed.

## REFERENCE

- (1) Lipschitz, W. L., Hadidian, Z., and Kerpcsar, A., *J. Pharmacol. Exptl. Therap.*, 79, 97 (1943).

# The Determination of Eucalyptol by Residual Titration With Hydrogen Bromide in Acetic Acid\*

By MARTIN I. BLAKE† and GILBERT RABJOHN

Eucalyptol is determined by treating samples with an excess of hydrogen bromide reagent. After a forty-eight-hour standing period, excess hydrogen bromide is titrated with sodium acetate in acetic acid. Mixtures of eucalyptol with thymol, menthol, and camphor are analyzed for eucalyptol content. Quantitative recoveries are reported.

THE OFFICIAL compendia have never recognized an assay procedure for eucalyptol as the free compound, or in the form of eucalyptus oil, or when combined with other medicaments. The British Pharmacopoeia (1) adopted a method of analysis based on the melting point of the molecular compound which forms with *o*-cresol. Guenther (2) describes a freezing point method. Martin and Harrisson (3) developed a spectrophotometric procedure based on the color reaction with *p*-dimethylaminobenzaldehyde. Methods for the detection and determination of eucalyptol were reviewed by these authors.

Durbetaki (4) used hydrogen bromide in acetic acid for the direct visual titration of oxirane oxygen in epoxy type compounds. The procedure was shown to be accurate and rapid. This paper describes a modification of this procedure for determining eucalyptol, an inner ether. The sample is treated with an excess of hydrogen bromide reagent and after the reaction is complete, unreacted hydrogen bromide is titrated with sodium acetate in acetic acid using methyl violet as the indicator. The procedure is applied to mixtures of eucalyptol with menthol, camphor, and thymol.

## EXPERIMENTAL

**Preparation and Standardization of Solutions.**—Hydrogen bromide 0.1 *N* in acetic acid was prepared (5) by adding bromine dropwise into tetrahydronaphthalene (Tetralin). The hydrogen bromide gas, which formed as a result of the reaction, was passed into glacial acetic acid. A concentrated solution of hydrogen bromide in acetic acid was prepared in this way and corresponded to approximately a 0.5 *N* solution. Sufficient glacial acetic acid was added to make the solution about 0.1 *N*. The final solution was standardized by titration with 0.1 *N* sodium acetate in acetic acid. This was accomplished by transferring exactly 25 ml. of

hydrogen bromide solution by pipet to a 125-ml Erlenmeyer flask. Twenty milliliters of glacial acetic acid was added, followed by the addition of three drops of methyl violet indicator solution (prepared by dissolving 1.0 Gm. methyl violet crystal in 100 ml. of glacial acetic acid). A stirring bar was added and the solution, magnetically stirred, was titrated to the first permanent blue color with 0.1 *N* sodium acetate in acetic acid.

Sodium acetate 0.1 *N* solution was prepared and standardized as described in an earlier paper (6).

**Procedure of the Analysis of Pure Eucalyptol.**—A sample of eucalyptol, 100–200 mg., was accurately weighed into an iodine flask. This was best accomplished by placing eucalyptol into a half-ounce dropper bottle and weighing the system. Eucalyptol, 5–8 drops, was added to the flask and the system was again weighed. The sample weight was obtained from the difference in weight. Twenty milliliters of glacial acetic acid was added to the flask, followed by the addition of exactly 25 ml. of 0.1 *N* hydrogen bromide solution. The flask was tightly stoppered and permitted to stand at room temperature for forty-eight hours. Excess hydrogen bromide was determined by rapidly titrating with 0.1 *N* sodium acetate solution to the first permanent blue color using three drops of methyl violet indicator solution for detection of the end point. The solution was magnetically stirred during titration. A blank run was conducted in the same manner except no eucalyptol was added. With each series of 4–6 determinations a blank was run to correct for any changes in normality of the hydrogen bromide solution. The analysis of pure eucalyptol is reported in Table I.

TABLE I.—ANALYSIS OF PURE CONSTITUENTS<sup>a</sup>

Constituent	Recovery, %
Eucalyptol	99.86 ± 0.11
Camphor	00.00
Thymol	00.00
Menthol	00.00

<sup>a</sup> Eight determinations

**Calculation of Per Cent Recovery.**—Per cent recovery of eucalyptol was calculated from the expression

$$\frac{\left[ \text{Vol. 0.1 } N \text{ acetate consumed in blank} - \text{Vol. 0.1 } N \text{ acetate consumed in run} \right] \times 7.713 \times 100}{\text{Weight of sample in mg.}} = \% \text{ Eucalyptol}$$

**Reaction Time.**—The course of reaction between eucalyptol and hydrogen bromide was studied by analyzing a series of eucalyptol samples after stand-

\* Received September 18, 1959, from the School of Pharmacy, North Dakota Agricultural College, Fargo.

† Present address: Chemistry Division, Argonne National Laboratory, Lemont, Ill.

The authors gratefully acknowledge Fritzsche Brothers, Inc., for financial support in this project.

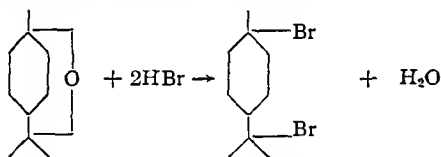
ing for varying time intervals. Reaction time varied from one to one hundred and twenty-eight hours. Reaction was found to be complete after standing at least forty hours. Forty-eight hours was selected as convenient time for this procedure.

**Analysis of Eucalyptol Mixtures.**—Constituents commonly employed with eucalyptol were analyzed in the manner described for eucalyptol. These included thymol, menthol, and camphor. Results are reported in Table I.

Mixtures containing eucalyptol and one or more other constituents were analyzed. These were prepared by weighing calculated quantities of the components into a 50-ml. volumetric flask and diluting to the mark with anhydrous methanol. Analysis was effected by transferring a 1-ml. aliquot of the solution by pipet to an iodine flask and treating in the same manner as the pure eucalyptol samples. At least four 1-ml. aliquots and a blank were run simultaneously on each mixture. Representative mixtures are indicated in Table II. The components are listed in the first column and their concentrations in mg. per ml. are shown in column 2. Per cent recoveries are indicated in the third column.

## DISCUSSION

Eucalyptol has been shown to react quantitatively with hydrogen bromide in acetic acid. The equation for the reaction appears to be



Since the reaction was found to be a slow one, direct titration was not feasible. However, good results were obtained by adding an excess of hydrogen bromide reagent and permitting the reaction to stand for a forty-eight-hour period. The excess hydrogen bromide was readily determined by titration with sodium acetate in acetic acid using methyl violet as the indicator. No difficulty was encountered in observing the first blue color denoting the end point. Since each mole of eucalyptol reacts with two moles of hydrogen bromide, the equivalent weight of the former is one-half the molecular weight or 77.13. Therefore, as shown in the expression for calculating per cent eucalyptol, each ml. of 0.1 *N* sodium acetate is equivalent to 7.713 mg. of eucalyptol. Quantitative recovery is shown in Table I. Since no reaction was observed with thymol, menthol, or camphor, the procedure described in this paper is suitable for determining eucalyptol in the presence of these constituents. Results of the analysis of a variety of such mixtures are shown in Table II.

TABLE II.—ANALYSIS OF MIXTURES FOR EUCALYPTOL CONTENT

Constituents in Mixture	Concentration, mg./ml.	Eucalyptol Recovery, %
Eucalyptol	138.0	99.12 ± 0.22
Camphor	50.4	
Eucalyptol	140.3	101.33 ± 0.31
Thymol	51.5	
Eucalyptol	139.5	99.01 ± 0.16
Menthol	49.0	
Eucalyptol	152.8	98.75 ± 0.25
Menthol	20.0	
Camphor	19.9	
Eucalyptol	151.6	99.45 ± 0.23
Menthol	40.2	
Thymol	39.8	
Eucalyptol	139.3	98.86 ± 0.19
Menthol	20.2	
Camphor	19.8	
Thymol	20.0	
Eucalyptol	143.4	101.85 ± 0.12
Menthol	39.8	
Camphor	40.1	
Thymol	41.2	

The procedure was applied to the analysis of eucalyptus oil for eucalyptol content. A series of ten determinations, based on the procedure described for eucalyptol, yielded an average result of 85.21%. However, by the British Pharmacopoeia method (1) an average result of 71.5% was obtained. The freezing point method, described by Guenther (2), gave an average result of 72.0%. Apparently, other constituents present in the oil, probably unsaturated compounds, reacted with the hydrogen bromide, producing high results. Other work conducted in this laboratory indicated that this reagent reacts quantitatively with certain unsaturated volatile oil constituents. This will be reported in another paper.

The hydrogen bromide titrant must be stored in a tightly stoppered container. It was noted that over a period of several weeks, the normality of the solution decreased slightly. This was taken into account by conducting a blank with each series of determinations.

The procedure described in this paper is a simple and accurate one. The chief disadvantage is the forty-eight-hour standing period required for completion of the reaction. The reaction appears to be very selective, affecting specific types of ethers and certain unsaturated compounds. Basic compounds such as amines also interfere.

## REFERENCES

- (1) "British Pharmacopoeia," General Medical Council, London, 1958, p. 879.
- (2) Guenther, E., "The Essential Oils," Vol. 1, D. Van Nostrand Co., New York, N. Y., 1948, p. 294.
- (3) Martin, E. W., and Harrison, J. W. E., *THIS JOURNAL*, 39, 677(1950).
- (4) Durbetaki, A. J., *Anal. Chem.*, 28, 2000(1956).
- (5) Duncan, D. R., "Inorganic Syntheses," Vol. 1, McGraw Hill, New York, N. Y., 1939, p. 151.
- (6) Blake, M. I., *THIS JOURNAL*, 46, 163(1957).

# The Toxicity and Safety Testing of Disposable Medical and Pharmaceutical Materials\*

By JOHN H. BREWER and HAROLD H. BRYANT

Many disposable surgical and pharmaceutical materials are not adequately tested for their toxicity and compatibility with tissues. Suggested techniques are described for testing these items. By the use of such tests the various items available may be checked and a choice made between those which should not be used and those which have been adequately tested and are found to be satisfactory.

**B**ECAUSE of high labor cost and an inadequate supply of properly trained personnel, the demand for disposable, ready-to-use medical and pharmaceutical materials has resulted in the introduction of many such items in the commercial market. Disposable syringes, needles, vials, catheters, drain tubes, and countless other items are now available. For the most part these are made of plastic or one of the newer, lighter weight alloys. Many of these items offer advantages over the original re-usable item. The throw-away hypodermic needle, for instance, cannot transfer infectious hepatitis from one patient to another, and, since the needle point does not have to withstand repeated injection, it can be made much sharper. More care can be given to its sterilization in autoclaves or gas sterilizers with the proper controls than by the busy physician with an office sterilizer. Also, the individual needle can be sealed in the final package before sterilization, and it maintains sterility until the time of use.

Other disposable items have similar advantages over the original re-usable ones. By changing the plasticizers or other chemicals in the plastic formulations, catheters may be made with any flexibility desired. From a pharmaceutical point of view, bottles and pharmaceutical "glassware" which will not break have many advantages. The pharmacist must take care, however, in the use of these materials, since some of the plasticizers may be leached out and react with the drugs stored in them. Or they may be permeable to, or soluble in, the solvents which they contain.

The individual plastic or alloy formulations

must be carefully tested by chronic and acute testing methods before being employed. Tests which simulate actual conditions of use should be employed, with added tests to give a large degree of safety.

The responsibility of the manufacturer does not end with the careful checking of the original formulation, but tests on each individual lot must be performed, since plasticizers, coloring agents, and plastics may change from lot to lot without the manufacturer's knowledge.

The Food and Drug Administration has been very interested in those plastic formulations used for food containers, and a number of publications have appeared stating which plasticizers and other substances may or may not be used for such items. This has led to some false sense of security on the part of the surgical suppliers; they felt that since their formulations met these requirements that they were entirely satisfactory and met all the requirements for medical use. A great deal of harm may have been done by the labeling of some plastic tubings as medical grade tubing, without adequately testing the material by imbedding samples in animals. Drains and indwelling catheters made from some of this material cause tissue reaction and necrosis which is open to bacterial infection.

Although the U. S. P. has certain sterility and toxicity requirements for plastic tubing assemblies used in blood collection and transfusion, no such requirements or tests are outlined by either the U. S. P. or FDA for tubing to be used for drains or indwelling catheters.

One has only to recall the reports in publications of the AMA on reactions to improperly tested plastic formulations used in watch bands, belts, etc., to understand how much more important is the adequate testing of plastics for surgical and medical use.

Several years ago the authors were asked by a firm, which was planning to manufacture a plastic-hubbed hypodermic needle, to develop toxicity and safety tests for this item. The procedure devised would be used to determine whether the plastic formulations, which met their physical specifications, would also prove nontoxic under all the uses to which needles would be subjected. Several arbitrary tests were developed; some

\* Received August 21, 1959, from the Biological Research Laboratories, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

This report deals with the development of routine methods which are applied to products of the Becton, Dickinson Co., Rutherford, N. J., and is reported with the permission of that Company.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

actually simulated conditions of use and others were developed to give a margin of safety. It was found that a few coloring materials could not be used since they would leech out on long contact with saline, which might occur during use. Some formulations were found to be toxic and could not be used. In like manner, some plasticizers were found to be unsatisfactory.

### TEST PROCEDURES

The specific tests which are applied to needles are as follows: Each needle hub formulation is checked, using the following solvents: 0.9% saline, 5.0% ethyl alcohol, sesame oil, and polyethylene glycol, since these would represent the most common solvents used in parenteral products. Not less than 20 needles are covered with each of the above menstrua, 1.5 ml. per needle. The overlay preparations, along with control portions of the four solvents, are held at 37° for eighteen to twenty-four hours. The eluates are decanted after vigorous agitation to aid elution of possible surface reaction products. The following tests are then performed:

**Acute Toxicity.**—Fifteen healthy mice, weighing 18–22 Gm., are injected with each of the menstrua, paralleled by five mice using the control solutions. The saline and ethyl alcohol eluates are injected intravenously, 1.0 ml. per mouse. The sesame oil and polyethylene glycol are injected intraperitoneally, 1.0 ml. per animal of the first, while a dilution of the polyethylene glycol is used which is just below the level causing toxic manifestations in previous control studies. The animals are observed for seventy-two hours.

**Bleb Reactivity.**—Each of the four elution menstrua is injected intracutaneously, 0.2 ml. into each of ten sites, paralleled by ten 0.2-ml. control blebs in the backs of two rabbits. The first three are injected undiluted, while the polyethylene glycol is diluted to contain 20 mg. in the 0.2-ml. bleb volume. The sites are observed for seventy-two hours for erythema, edema, or necrosis.

**Pyrogenicity.**—Ten needles are heated in 200 ml. saline to not less than 85° for one hour and the U. S. P. pyrogen test on the decanted eluate performed.

After the original formulation study, production control tests are run, using the saline overlay only.

To simulate actual conditions of use, as in dermatological work, needles are attached to a small syringe and 0.25 ml. saline is drawn into the syringe, leaving the needle cannula filled. These are placed in a tray and held twenty-four hours at 37°, and then blebs raised on a rabbit. Since only a very small area of the inside of the hub is actually in contact with the solution, one might never expect to obtain any toxic reaction. In this manner it has been possible to observe toxicities on some of the formulations discussed above.

### OBSERVATIONS

Although these tests were designed for plastic hubs, some interesting findings were noted when experimental lots of aluminum hub needles were submitted to these tests. At first it was not

thought necessary to test an all metal needle for toxicity, since no plasticizers or epoxy resins were involved. Since it was known that some of the alloys used contained a small amount of lead to facilitate manufacture, we decided to submit these needles to the same test. A floccular precipitate developed in the saline, and on intravenous injection into the mice killed all of the ten mice within a few minutes. When this floccular material was submitted to the bleb type of test, all the test sites gave positive skin reactions.

These sudden deaths and positive skin tests could not possibly be due to the small amount of lead eluted from these hubs since less than 1 mg. of weight loss occurred during the saline soaking. The deaths must be due to embolism of the floccular material which occurs with aluminum and saline. These tests were repeated with distilled water, and only a slight haze was seen. However, when this material was made isotonic with sodium chloride for intravenous injection, the floccular precipitate developed and death resulted in the mice, although not to the same extent since the amount of precipitate was not as great. No deaths occurred when this material was given intraperitoneally or when the experimenter was very careful not to disturb the precipitate and only injected the supernatant liquid.

This type of safety test does not simulate actual conditions of use since the entire surface area is in contact with the solution. In the actual use type of test in which the needles were placed on the syringe and 0.25 ml. saline drawn into the syringe and allowed to stand, as might occur in a dermatologic clinic where large numbers of allergens are being tested, positive skin tests did occur in the blebs raised on the rabbits, provided the material was mixed by shaking and the precipitate which formed expelled. To determine whether or not this material might also cause false positive reactions in humans, dermatologists repeated these tests, making sure that they mixed the material before injection. About 40% false positive reactions on two series of tests employing this technique were obtained. The number of reactions in the animals and humans is proportional to the amount of precipitate which is injected. If the syringe is handled very carefully so that most of the precipitate remains in the syringe, fewer positive reactions will result.

From the point of view of the hospital pharmacist or laboratory technician, aluminum-hubbed needles should not be used on a pitkin or other type dispensing syringe, which is placed back in the flask of saline each time in order to fill it and then remains in the saline. When used in blood grouping or other tests employing washed red cells, the small amount of aluminum eluted may cause agglutination of the red cells and lead to erroneous results. This finding was noted by one of the blood banks that was using saline from a bottle to which an aluminum needle was attached and which remained in contact with the saline for a long period of time.

For disposable syringes a slightly different technique must be employed in testing. Each syringe formulation is studied, using the solvents discussed under needle testing. With the syringes, the barrels of 20 units are filled with the solvent and allowed to warm to 37° for twenty-four hours. The eluates are then combined and tests for acute toxicity and



Fig 1 —Subcutaneous implant, seventy two hours, no reaction



Fig 2 —Subcutaneous implant, seventy-two hours, showing reaction



Fig 3 —Intramuscular implant, seventy-two hours, no reaction

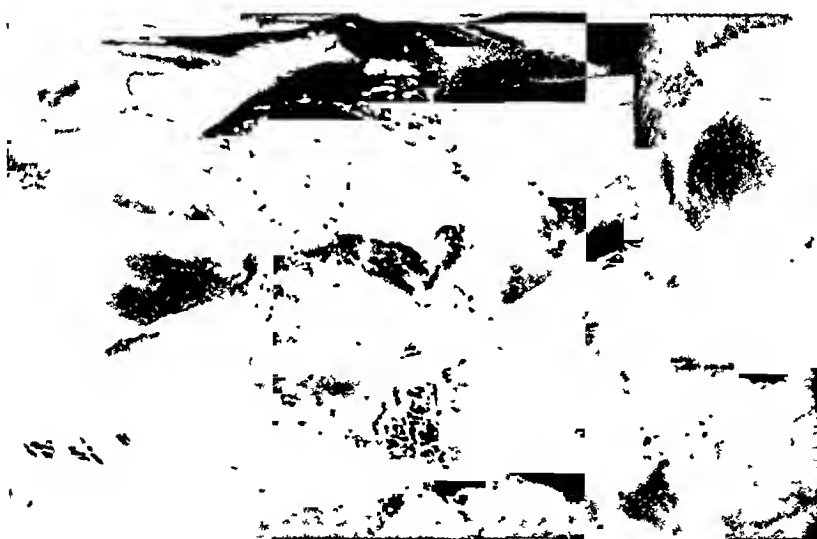


Fig 4 —Intramuscular implant, seventy-two hours, showing reaction



bleb reactivity simulating those for the needle eluates are performed.

Plastic syringe plungers and rubber plunger tips are studied by elution, in much the same manner as described above for needles, except that 10 Gm. of the test material may be covered with 50 ml. of the elution menstruum rather than by using 20 test items.

Control testing of approved formulations in syringe assemblies is performed by drawing 1.0 ml. of 0.9% saline into the syringe plus 1.0 ml. of air, and eluting the assembly for eighteen to twenty-four hours at 37°. Twenty units are so eluted and the eluates combined for acute toxicity and bleb reactivity tests.

Other plastic items: indwelling catheters, drains, stomach tubes, intravenous feeding tubes, and tubing for general medical use are also tested. Plastic formulations developed for use in contact with body tissues, especially where the normal epithelium has been interrupted, are tested for acute toxicity, bleb reactivity, tissue reaction, and pyrogenicity.

Ten grams of the material is cut into relatively small pieces and covered with 50 ml. of sterile 0.9% saline; a like preparation is made using 5.0% ethyl alcohol. These overlays are then warmed to 37° for twenty-four hours, along with control portions of the two menstrua. The overlays are then vigorously shaken and the elution menstruum decanted.

Acute toxicity is determined by injecting the undiluted eluates and control solution in doses up to 1.0 ml., both intravenously and intraperitoneally, in mice weighing 18-22 Gm. This is equivalent to injecting the eluate from 10 Gm. of the plastic per Kg. of body weight.

Bleb reactivity is determined in a manner simulating that described above under needle testing.

Tissue reaction or toxicity exhibited by the tissues in contact with the plastic material is studied by implanting sections roughly 1.5 × 10 mm. in size subcutaneously and intramuscularly in the rabbit. At least four such sections are implanted subcutaneously, paralleled by four known nonreactive controls; a like number are implanted intramuscularly in a second animal. The implants are allowed to remain in place for seventy-two hours, after which the animals are sacrificed and the implant sites examined for surrounding reactive material. The acute, seventy-two-hour tests are evaluated by careful gross examination only. Those formulations which pass the acute implant tests and which

may be in contact with the body tissues for a longer period of time are also implanted for sixty-day periods. These implant sites are then evaluated by gross examination and histologically by a competent clinical pathologist. Likewise, those formulations likely to be used in contact with the central nervous system are implanted for seventy-two hours and sixty days in the cerebral cortex of the rabbit for careful evaluation. The implantation procedure has proved to be the most useful of the tests devised to determine tissue compatibility of a plastic formulation.

Pyrogenicity is studied by heating 10 Gm. of the formulation in 200 ml. of saline to not less than 85° for one hour, and subjecting the eluate to the standard U. S. P. pyrogen test procedure.

When a number of the medical grade tubings were tested, some were found to be entirely satisfactory and produced no reaction in contact with the tissues, as shown in Figs. 1 and 3. Other samples of tubing produced a very severe reaction, as shown in Figs. 2 and 4.

## SUMMARY AND CONCLUSIONS

It is felt that before use of any material which may produce pathological conditions, that unless there is proof that such testing has been done on each lot produced, similar tests on each lot of material purchased should be run.

As stated above, some of these tests are severe and do not simulate actual conditions of use, but were designed to give a margin of safety. They may also explain some of the reactions which have been encountered in the use of these "throw-away" type products which, until now, could not be accounted for.

It is realized that the tests described are not ideal for all of the different disposable items available. The purpose of this paper is to stimulate the hospital pharmacist or other persons responsible for the purchase of disposable medical supplies to make sure that adequate sterility and toxicity testing has been performed, and that the items are satisfactory for the purpose for which they are to be employed.

# Effects of Tranquilizers on Bacterial Toxemias II.

## Meprobamate\*

By LEO GREENBERG and JAMES W. INGALLS

Pretreatment of rats and mice with meprobamate prior to inoculation with lethal doses of bacterial exotoxins and endotoxins resulted in a highly significant prolongation of life. However, the drug showed no effect on survival time in overwhelming infections induced by *Diplococcus pneumoniae* or *Salmonella typhimurium*. In *Salmonella enteritidis* infection, chlorpromazine increased the susceptibility of mice far more markedly than did meprobamate.

**M**EPROBAMATE is a potent pharmacological agent of wide clinical use, and its role in the alleviation of stress syndromes of various etiologies is well known. Yet, few references to its role in bacterial stress have been reported. It is known that the drug is capable of significantly prolonging the life of guinea pigs in diphtheric toxemia (1), and recent publications have indicated that it significantly affects the immunological response of rabbits to *Salmonella typhimurium* infection (2, 3).

In a previous study (4), it was shown that reserpine is capable of favorably influencing the survival time of experimental animals injected with lethal doses of tetanus toxin, botulinum toxin, or *Diplococcus pneumoniae*. On this basis, our experiments were extended to other psychotropic drugs within the framework of bacterial stress. The present paper summarizes our results with meprobamate.

### MATERIALS AND METHODS

The animals used in this study were 200-300 Gm. male and female CFN rats and 20-25 Gm. CF1 male mice. All were caged in small groups and kept in the thermostatically controlled animal house for several days prior to use. In toxin experiments, food was withheld after inoculation, but water was given *ad libitum*.

Meprobamate<sup>1</sup> powder was dispersed in 2.5 or 5.0% acacia solution. In all experiments, paired groups of animals were used. The control group was injected with acacia solution in a volume equal to that received by the experimental group. All control and experimental animals in any given series were inoculated within a few minutes of one another.

Tetanus toxin<sup>2</sup> in 0.85% sodium chloride solution with 0.3 M glycine, an Lf titer of 870/ml. and a

mouse LD<sub>50</sub> of 112 million was used throughout. Purified *Clostridium botulinum* type A toxin<sup>3</sup> diluted with two parts of glycerol and a mouse LD<sub>50</sub> of approximately  $5 \times 10^6$ /ml. was used. Toxins were stored in the refrigerator, the botulinus material at -20° to maintain potency. *Escherichia coli* 026:B6 and *Salmonella typhosa* 901 endotoxins were purchased as powdered lipopolysaccharides from Difco Laboratories. All toxins were diluted with sterile isotonic saline.

*Diplococcus pneumoniae* strain SVI was obtained as a lyophilized culture from the Rockefeller Institute and grown in brain heart infusion broth (Difco). Slants were maintained on brain heart infusion agar with added blood. Subculturing was done weekly and virulence maintained by monthly mouse passage. For inoculations, eighteen-hour broth culture containing approximately  $2 \times 10^8$  organisms/ml. was used.

*Salmonella enteritidis* (No. 13076) and *Salmonella typhimurium* (No. 6994) cultures were purchased from the American Type Culture Collection and maintained on Trypticase soy agar (Difco) with added 1% glucose. An eighteen-hour Trypticase soy broth culture containing approximately  $4 \times 10^8$  organisms/ml. was used for inoculations.

All toxin and bacterial injections were in a volume of 0.1 ml. All inoculations were made intraperitoneally. None of the diluent substances used showed any toxicity to mice or rats, and sterile broth injections were without apparent effect.

### RESULTS AND DISCUSSION

**Toxin Studies.**—Results with meprobamate at various dose levels in both rats and mice subjected to lethal doses of bacterial toxins are summarized in Table I. It is evident that in doses between 200 mg./Kg. and 533 mg./Kg., meprobamate exerted a powerful life-prolonging effect in both species. This effect was demonstrated in both exotoxic and endotoxic states, and in all cases was directly proportional to the dose of meprobamate used.

As in our previous findings with reserpine (4), this response was evoked only under the specific set of conditions which were employed, namely, a large dose of meprobamate injected shortly before the introduction of a highly lethal dose of toxin. The failure of the drug to influence survival when administered one hour post-toxin is apparent. Further, no influence on survival was noted following the use of highly dilute toxin solutions and the use of small divided doses of meprobamate prior to toxin injection.

The studies on *E. coli* and *S. typhosa* endotoxins were carried out several weeks apart. The remarkable similarity of the data achieved apparently sup-

\* Received March 14, 1960, from the Research Institute of the Brooklyn College of Pharmacy, Long Island University, Brooklyn 16, N. Y.

<sup>1</sup> Supplied as Miltown through the courtesy of Wallace Laboratories, New Brunswick, N. J.

<sup>2</sup> Supplied through the courtesy of Dr. H. A. Dettwiler, Eli Lilly & Co., Indianapolis, Ind.

<sup>3</sup> Supplied through the courtesy of Matteo Cardella, Immunology Branch, U. S. Army Biological Warfare Laboratories, Fort Detrick, Frederick, Md.

TABLE 1.—EFFECT OF MEPROBAMATE ON SURVIVAL TIME FROM BACTERIAL TOXINS IN MALE RATS AND MICE

Animal	No.	Treatment	Dilution	Survival, min. $\pm$ S. E.	Significance
Botulinus Exotoxin					
Mice	10	533 mg./Kg. Meprobamate	1-100	135.0 $\pm$ 11.9	$P < 0.05$
Mice	10	Acacia solution	1-100	102.2 $\pm$ 8.9	
Mice	25	533 mg./Kg. Meprobamate	1-10	92.0 $\pm$ 7.9	$P < 0.01$
Mice	25	Acacia solution	1-10	65.9 $\pm$ 2.9	
Rats	7	500 mg./Kg. Meprobamate	1-10	515 $\pm$ 50	$P < 0.01$
Rats	7	Acacia solution	1-10	348 $\pm$ 15	
Rats	10	400 mg./Kg. Meprobamate	1-10	437 $\pm$ 28	$P < 0.01$
Rats	10	Acacia solution	1-10	323 $\pm$ 11	
Rats	10	400 mg./Kg. Meprobamate post-toxin	1-10	255	No significance
Rats	10	Acacia solution post-toxin	1-10	258	
Tetanus Exotoxin					
Mice	10	533 mg./Kg. Meprobamate	1-100	234.5 $\pm$ 20.7	$P < 0.05$
Mice	10	Acacia solution	1-100	180.0 $\pm$ 12.7	
Rats	10	500 mg./Kg. Meprobamate	1-10	1057 $\pm$ 122	$P < 0.02$
Rats	10	Acacia solution	1-10	719 $\pm$ 33	
Rats	10	400 mg./Kg. Meprobamate	1-10	997 $\pm$ 62	$P < 0.001$
Rats	10	Acacia solution	1-10	719 $\pm$ 33	
Rats	8	200 mg./Kg. Meprobamate	1-10	830 $\pm$ 37	$P < 0.05$
Rats	8	Acacia solution	1-10	743 $\pm$ 9	
Rats	8	500 mg./Kg. Meprobamate post-toxin	1-10	1227 $\pm$ 234	No significance
Rats	8	Acacia solution post-toxin	1-10	1103 $\pm$ 238	
E. coli Endotoxin					
Mice	10	533 mg./Kg. Meprobamate	1-50	865 $\pm$ 81	$P < 0.02$
Mice	10	Acacia solution	1-50	622 $\pm$ 38	
S. typhosa Endoxotin					
Mice	10	533 mg./Kg. Meprobamate	1-50	861 $\pm$ 79	$P < 0.02$
Mice	10	Acacia solution	1-50	624 $\pm$ 35	

ports the current concept of the nonspecific nature of such lipopolysaccharide extracts from Gram-negative bacilli.

**Bacterial Studies.**—It has been previously shown (4) that reserpine is capable of significantly prolonging the life of animals injected with an overwhelmingly lethal dose of *Diplococcus pneumoniae*. Experiments with meprobamate and *D. pneumoniae* did not yield conclusive results although almost a thousand animals were used in an effort to establish a consistent response.

With our bacterial inoculum, infected animals always died between the sixteenth and forty-eighth hours postinjection. A single dose of 420 mg./Kg. meprobamate either one hour before or one hour after *D. pneumoniae* challenge did not affect survival. Preconditioning the animals with four injections of 300 mg./Kg. meprobamate eight hours apart prior to bacterial inoculation had no effect upon survival, and injections of the drug on the same schedule commencing immediately after bacterial inoculation tended to shorten survival. A single injection of 720 mg./Kg. meprobamate one hour prior to bacterial introduction resulted in increased survivals, while a dose of 800 mg./Kg. reduced survivals as compared to controls. In neither case, however, was statistical significance achieved.

During our experiments, we noted with interest the report by Grosz and Norton (5) that chlorpromazine increased the susceptibility of mice to infection with *Salmonella enteritidis*. Therefore, we compared the relative intensity of this response in both chlorpromazine and meprobamate-treated animals inoculated with *S. enteritidis*. From the

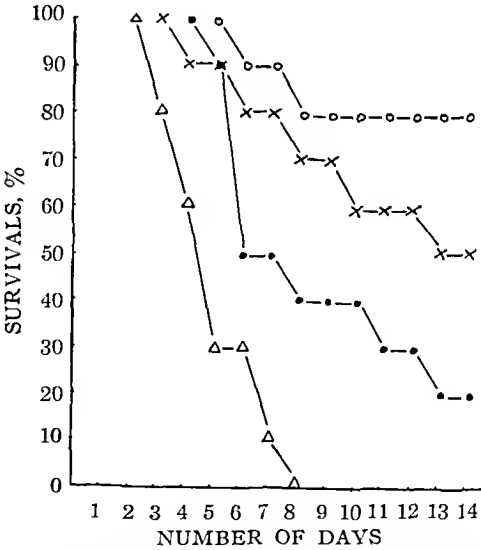


Fig. 1.—Effect of meprobamate and chlorpromazine on survival time of mice infected with *Salmonella enteritidis*. O Acacia, meprobamate control; X water, chlorpromazine control; ● meprobamate, 533 mg./Kg.; Δ chlorpromazine, 2 mg./Kg.

results plotted in Fig. 1 it is evident that chlorpromazine did significantly shorten the life of infected animals in the dose employed. Meprobamate also reduced the survival time of infected animals, but to a far lesser extent than did chlorpromazine.

Similar comparisons were then undertaken using *Salmonella typhimurium* as the stress organism. In several trials, no significant variations from control values were demonstrated either by chlorpromazine or meprobamate treatment. From these results, we conclude that the enhancement of lethality by chlorpromazine noted by Grosz and Norton, and the enhancement of lethality by both chlorpromazine and meprobamate noted by us is not a universal response to bacterial stress.

## SUMMARY AND CONCLUSIONS

1. Mice and rats were subjected to overwhelming bacterial stress utilizing purified exotoxins, lipopolysaccharide endotoxins, and broth cultures.

2. Pretreatment with meprobamate exerted a significant life-prolonging effect in *Clostridium tetani* and *Clostridium botulinum* exotoxemias in both mice and rats. This response was correlated to the dose of the drug employed.

3. Pretreatment with meprobamate resulted in a significant prolongation of life in mice

inoculated with lethal doses of *E. coli* and *S. typhosa* endotoxins.

4. Various dosage schedules of meprobamate were evaluated in overwhelming *D. pneumoniae* infection. No significant effects on survival were demonstrated.

5. Meprobamate enhanced the lethality of *S. enteritidis* in mice, but to a lesser extent than chlorpromazine. Neither meprobamate nor chlorpromazine significantly altered the lethality of *S. typhimurium* for mice.

6. In laboratory animals, the antistress spectrum of meprobamate extends beyond emotional stress into the physiological stress of bacterial toxemias.

## REFERENCES

- (1) Greenberg, L., and Ingalls, J. W., *Nature*, 184, 1721 (1959)
- (2) Compagnucci, M., Ferlazzo, A., and Francesconi, G., *Boll. soc. ital. biol. sper.*, 35, 313 (1959)
- (3) Ferlazzo, A., Alosi, C., and Lombardo, G., *ibid.*, 35, 315 (1959)
- (4) Greenberg, L., Ingalls, J. W., and Zupko, A. G., *THIS JOURNAL*, 49, 225 (1960).
- (5) Grosz, H. J., and Norton, J., *Science*, 129, 784 (1959).

# Investigation of *Machilus macrantha* Nees. I\*

## Pharmacognostical and Phytochemical Study of Root

By K. N. GAIND and S. K. BAVEJA

The root of *Machilus macrantha* Nees. has been studied for physical characteristics, histology, extractives, ash, and alkaloidal constituents. Anatomical characteristics include: a continuous band of small stone cells constituting phelloderm; a pericycle consisting of large stone cells; isolated groups of stone cells in the phloem; lignified phloem and xylem fibers with narrow and large lumen, respectively; copious mucilaginous matter in the phloem parenchyma cell walls; and oil cells in cortex, phloem, and xylem. The extractives showed the presence of phytosterols, glycosides, and volatile oil. From the limed drug, extraction and isolation of a base as hydrochloride, provisionally named "machiline hydrochloride," melting with decomposition between 248–250°, two alkaloids as picrates and one reineckate are described. The yield of machiline hydrochloride was highest (0.0725 per cent) from the root collected in the month of May. Paper chromatography of the extract revealed the presence of at least five alkaloids.

THIS STUDY was prompted by the information that the root of *Machilus macrantha* Nees. is considered useful in hypertension by the practitioners of the Indian indigenous system of medicine. It has been reported in the literature (1–3) that the bark of this plant is used in con-

sumption, asthma, and rheumatism, and its leaves are applied to ulcers.

No prior investigation of this plant has been reported. From another species of this genus, *M. thuibergii*, an oil was extracted and its constants and constituents were studied (4).

This study was made to provide sufficient pharmacognostical characterization for the identification of *M. macrantha* root and to establish a scientific basis for its use in hypertension.

\* Received September 10, 1959, from the department of Pharmacy, Panjab University, Chandigarh, India.  
The authors are sincerely grateful to Mr. R. S. Sandhu, Pharmacognocist, Medical College, Amritsar, for his assistance in pharmacognostical study.

## EXPERIMENTAL

**Materials Used.**—The roots and flowering shoots of *M. macrantha* were obtained from Kulathupuzha, Kerala State (India), through the courtesy of Mr T K Devakaran, forest range officer. It was authenticated by Mr. B. M. Raizada, Hcad, Division of Forest Botany, Forest Research Institute, Dehradun. The dried roots, pulverized to moderately coarse powder, were used for phytochemical work.

**The Plant.**—*Machilus macrantha* Nees of the *Lauraceae* family known as Gumara or Gumbara in Konkan; Uravu in Malayalam; Anaikkuru, Kollamavu, and Mullai in Tamil; and Ululu in Sinhalese, has been described previously (1, 5). It is a large tree attaining a height of 20–25 M and is fairly abundant from Konkan southwards in the Western Peninsula (India) and Ceylon.

**Physical Characteristics of Root.**—The roots examined (Fig. 1) were large pieces, 15–20 cm. in diameter, and small pieces, 3–3.5 cm. in diameter. They were cylindrical, rarely flattened, and light in weight when dry. Externally, they were yellowish-brown to dirty buff colored with longitudinal wrinkles. Some of the large pieces showed transverse cracks. In small pieces the transverse cracks were rare but transverse ridges and occasionally lenticels were present. They also showed rootlet scars. Internally, there was a narrow, granular, brown to dark brown bark, 3–3.5 mm. thick in small pieces and 7–10 mm thick in large pieces, and an inner pale brown to reddish-brown wood with no growth rings. The fracture of the bark was short, smooth toward the periphery and granular toward the inside; that of the wood was splintery. The dried root had a slight aromatic odor and a slimy taste. The bark, on soaking in water, ejected copious mucilaginous matter.

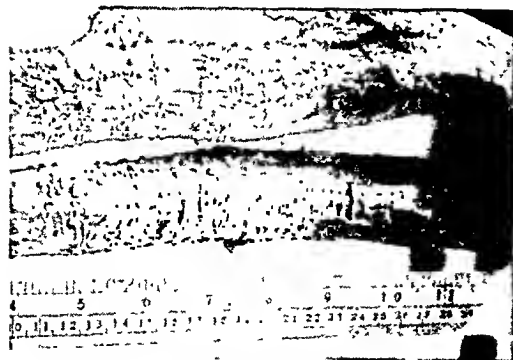


Fig. 1.—Root of *Machilus macrantha* Nees.

**Histology of *M. macrantha* Nees. Root.**—Roots of 3–3.5 cm. diameter were selected (see Figs. 2 and 3). Samples from these were prepared by the usual pharmacognostic methods (6). Sections were cut on a wood microtome and by free hand sectioning. Numerous temporary mounts and permanent mounts of transverse, radial longitudinal, and tangential longitudinal sections of the root specimens were made and examined microscopically. For study of individual cells, different tissues were macerated in potassium chlorate and nitric acid reagent (7) and slides

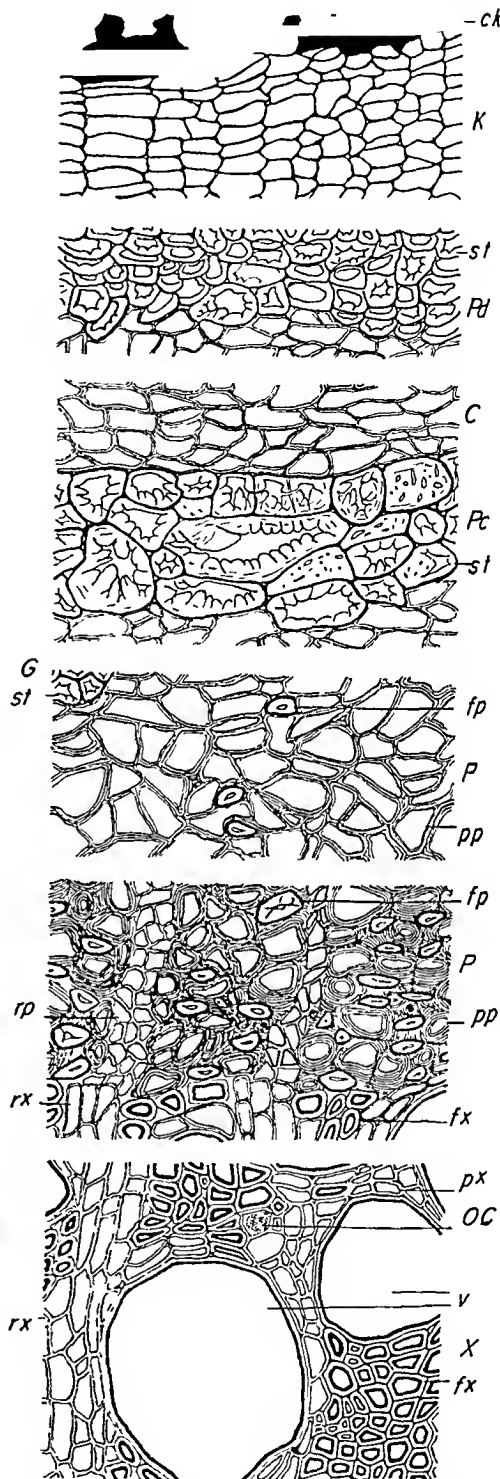


Fig. 2.—Transverse sections of representative portions of *Machilus macrantha* Nees. root. K, cork; Pd, phelloderm; C, cortex; Pc, pericycle; P, phloem; x, xylem; ck, compressed cork cells; st, stone cells; fp, phloem fibers; pp, phloem parenchyma; rp, phloem rays; px, xylem parenchyma; oc, oil cells; v, xylem vessels; G, st, group of stone cells; fx, xylem fiber; rx, xylem ray.  $\times 140$ .

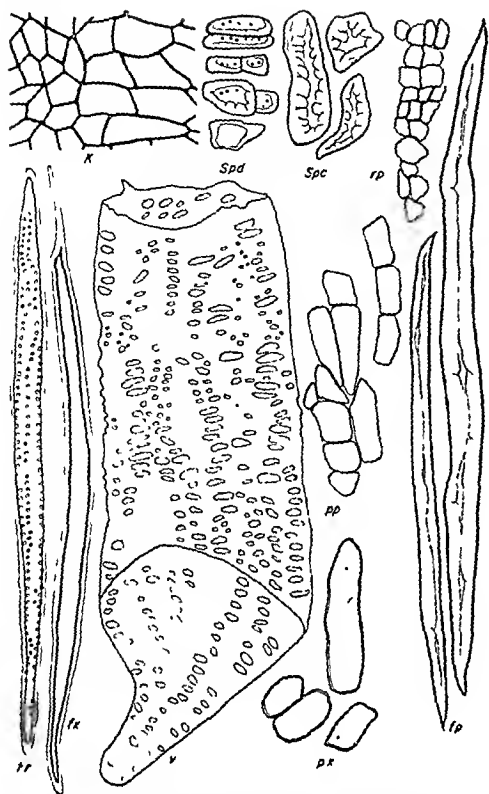


Fig. 3.—Histological elements occurring in macerated tissues and powdered root of *Macchilus macrantha* Nees. K, cork cells in surface view; *spd*, stone cells of phelloderm; *spc*, stone cells of pericycle; *rp*, phloem ray cells; *pp*, phloem parenchyma; *sp*, phloem fibers; *px*, xylem parenchyma; *v*, vessel with large and small pits; *fx*, xylem fiber; *tr*, tracheid.  $\times 100$ .

mounted in 50% glycerin. In the following description, the symbols R, T, and L refer to measurements made in radial, tangential, and longitudinal directions, respectively.

In a cross section 33 mm. in diameter, the bark occupied 7 mm. and the wood 26 mm. The cork zone was up to 25 layers of cells deep, covered on the outside by a few layers of collapsed cork cells. The cells were rectangular to somewhat squarish, arranged in more or less radial files; polygonal in tangential section; R, up to  $2.8\mu$ ; T,  $11.0\mu$ ; and L,  $80\mu$ ; with suberized walls. The phelloderm was composed of up to eight layers of somewhat rectangular to isodiametric stone cells, 15 to  $12.0\mu$ , arranged in radial rows corresponding to those of cork cells; cell walls thickened, usually along radial and interior tangential sides, yellow, lignified and traversed by a few simple pore canals; lumen comparatively large but variable and occluded with dark reddish-brown contents. The cortex parenchyma cell walls were somewhat thickened with mucilaginous matter. Reddish-brown contents and a few simple starch grains were present in some cells. A few yellow oil cells were interspersed in the cortex, phloem, and xylem parenchyma. The conspicuous pericycle was composed of three to five layers of stone cells of 5 to  $25\mu$ ; cell walls thick, yellow, lignified

with a few striations in some; traversed by funnel-shaped simple and branched pore canals; lumen narrow but variable, totally occluded in a few, and devoid of cell contents. The phloem comprised 75% of the thickness of the bark. The phloem rays traversed the inner two-thirds of the phloem, were usually up to three cells wide, rarely four, 15 to 25 cells deep longitudinally, somewhat wavy, tapering outwards, and in continuation with their counterparts in the xylem. The phloem parenchyma cell walls were thickened with mucilaginous matter, more so on cells lying interiorly, the thickening being reddish brown and striated. The phloem fibers were numerous, thicker and longer in the interior region, up to  $1,240\mu$  long and  $60\mu$  thick, with yellow lignified walls traversed by a few simple rarely branched pits; lumen narrow, partially obliterated; ends bluntly pointed, rarely sharp. A few small groups of yellow lignified stone cells with narrow lumen were also present. The primary xylem was represented by a group of xylem elements in the center. The secondary xylem had xylem rays of straight to curved character up to four cells wide and 20 cells deep longitudinally, with irregularly rectangular cells up to  $50\mu$  long. The rounded or somewhat oblong vessels occurred singly or in groups of two to four, with their adjacent walls flattened. They were up to  $860\mu$  long and  $300\mu$  broad. The vessel walls bore numerous pits of varying size, the larger ones being transversely or obliquely elongated. The wood parenchyma was scarce, the cells being usually elongated longitudinally; L, up to  $250\mu$ ; R and T,  $50\mu$ . A few tracheids up to  $1,100\mu$  long and  $50\mu$  broad with pitted walls were also present. The rest of the xylem was constituted of fibers having moderately thick, yellow, lignified walls and a relatively large lumen. They measured up to  $1,250\mu$  long and  $50\mu$  broad and were arranged together with xylem parenchyma and tracheids in more or less radial files. Intercellular spaces were present.

**Powdered *M. macrantha* Root.**—This was pale brown with a slimy taste and aromatic odor. Under the microscope it showed: numerous fragments of wood fibers with thick, yellow, lignified walls and comparatively large lumens; phloem fibers with narrow and at places obliterated lumens and thick, yellow, lignified walls bearing simple pits; stone cells isodiametric, elongated or irregularly shaped, single or in groups with lumens of variable size, and thick, yellow, lignified walls bearing simple and branched pits; fragments of vessels and tracheids with pitted walls; scattered fragments of cork, with polygonal cells having suberized walls; and a few simple starch grains  $0.5$ – $1.0\mu$ , conspicuous in Smith's starch reagent.

**Extractives.**—Root powder (15 Gm) was extracted successively with solvents in the order given below, employing a Soxhlet extraction apparatus, the preceding solvent being completely removed from the marc before starting extraction with the next solvent. The percentages of the extractives obtained were: petroleum ether ( $40$ – $60^\circ$ ), 2.38%; ether, 0.32%; chloroform, 0.27%; and absolute alcohol, 3.90%. Volatile oil was detected in petroleum ether, phytosterols in petroleum ether and ether, and glycosides in petroleum ether, ether, and chloroform extracts. Dilute hydrochloric acid

extract of root powder gave positive tests with alkaloidal reagents.

**Determination and Analysis of Ash.**—The methods used for determination of ash content were those of the British Pharmacopoeia 1958 (8). The results were as follows: total ash, 2.3%; water-insoluble ash, 1.78%; water-soluble ash (by difference), 0.52%; acid-insoluble ash, 0.235%; and acid-soluble ash (by difference), 1.545%. The total ash contained sodium, calcium, magnesium, nitrate, and carbonate.

**Extraction and Isolation of Machiline Hydrochloride.**—Root powder in 0.8-Kg. quantities was mixed with 10% of its weight of calcium hydroxide, moistened, allowed to stand for four hours, and dried at 60°. The limed drug was extracted with alcohol (95%) in a Soxhlet apparatus until exhausted of alkaloids. The alcohol was recovered from the extract and the residue kneaded with 2% hydrochloric acid in portions until the alkaloids were completely taken up in acid solution. The pH of the solution was adjusted to 4.0 with dilute ammonia solution and extracted with chloroform until the latter extracted no more alkaloids. The pH was then raised to 8.0 and extraction with chloroform was repeated. The aqueous solution was then made highly alkaline with 28% ammonia solution, extracted with chloroform, the chloroform solution dried over exsiccated sodium sulfate, the chloroform recovered, the residue taken up in dry ether, and dry hydrochloric acid gas bubbled through the ether solution. The precipitated alkaloidal mass, on repeated crystallization from absolute alcohol, gave white crystals, elongated rectangular plates (under the microscope), melting range 248–250° (decomposition). It did not give a positive test with the usual precipitating alkaloidal reagents. It has been provisionally named "machiline hydrochloride." The yield of machiline hydrochloride obtained from *M. macrantha* root, collected in different months was as follows: February, 0.056%; May, 0.0725%; August, 0.038%; and November, 0.031%. The bark of the root collected in August was separated. It constituted 21.5%, by weight, of the whole root and on extraction yielded 0.0045% of machiline hydrochloride; the wood yielded 0.0472%. Studies on the chemical constitution and pharmacological action of machiline hydrochloride are reported in Part II of this series.

**Isolation of Two Alkaloids as Picrates.**—From the mother liquor left after crystallization of machiline hydrochloride, the solvent was recovered and the alkaloids precipitated with picric acid in aqueous solution. The precipitated picrates were separated, dried in a vacuum desiccator, taken up in absolute alcohol, and chromatographed on a column of alumina. Of the five bands formed, the lowermost yellowish-brown band (fraction 1) and the next higher yellow band (fraction 2) were eluted with absolute alcohol, the eluted fractions concentrated and kept separately in desiccators containing, respectively, ether and benzene. From fraction 1, a microcrystalline brown precipitate melting with decomposition between 157–162° was obtained during one

week, and from fraction 2, yellow crystals in the form of elongated rectangular plates, melting with decomposition between 270–272°, were obtained in three to five days.

**Isolation of the Water-Soluble Alkaloid as a Reineckate.**—The highly alkaline aqueous liquid left after extraction of the chloroform-soluble alkaloids still gave a strong test for alkaloids. It was concentrated under reduced pressure, the separated ammonium chloride was filtered off, and the alkaloid precipitated by addition of 2% solution of ammonium reineckate in dilute hydrochloric acid. The precipitated complex was separated, dried, taken up in dry acetone, and passed through a column of alumina. The clear, bright orange-red eluate was concentrated and kept in a vacuum desiccator overnight. The orange-red crystals formed were separated, washed with ether, and dried. The compound melted with decomposition at 260–262°.

**Paper Chromatographic Analysis.**—The ascending method was employed. The apparatus consisted of a specimen jar, 40 cm. × 10 cm., fitted with a rubber stopper through which passed a glass rod bearing hooks at the lower end. Paper strips (30 cm. × 2 cm.) were cut from Whatman No. 1 filter paper and buffered at pH 4.0 by dipping in 5% aqueous solution of monosodium citrate, blotting, and drying at 60° for twenty-five minutes. The solvent system consisted of *n*-butanol:water:citric acid, 50:50:1 Gm.; the organic layer was employed as the mobile phase. Fresh solvent was necessary for each resolution to achieve reproducible  $R_f$  values. Samples for analysis consisted of the concentrated alcoholic extract and the residues of the chloroformic and aqueous fractions derived from it, redissolved in alcohol. Development was performed at 15° ± 2°. The chromatograms were air dried and dipped in Munier's modified Dragendorff's reagent (9), and  $R_f$  values were calculated. In all, 5 bands were formed on the different chromatograms, their average  $R_f$  values being 0.90, 0.71, 0.47, 0.25, and 0.08. The water-soluble alkaloid formed the lowermost band which gave a purple spot with the Munier's reagent while the others gave orange spots on yellow background. The two high  $R_f$  values spots were pale yellow to yellowish brown even before treatment with the modified Dragendorff's reagent.

## REFERENCES

- (1) Kirtikar, K. R., and Basu, B. D., "Indian Medicinal Plants," Vol. 3, Lalit Mohan Basu, Allahabad, India, 1933, pp. 2155–2157.
- (2) Chopra, R. N., "Indigenous Drugs of India," The Art Press, Calcutta, India, 1933, p. 505.
- (3) Chopra, R. N., Nayar, S. L., Chopra, I. C., "Glossary of Indian Medicinal Plants," Council of Scientific and Industrial Research, New Delhi, India, 1956, p. 159.
- (4) Takeshita, T., *Yushi Kagaku Kyokashu*, 2, 239 (1953).
- (5) Cook, T., et al., "Flora of the Presidency of Bombay," Vol. 2, Taylor and Francis, London, England, 1908, p. 536.
- (6) Johanson, D. A., "Plant Microtechnique," 1st ed., McGraw-Hill Book Co., New York, N. Y., 1940, p. 126.
- (7) Trease, G. E., "A Text Book of Pharmacognosy," 6th ed., Baillière Tindall and Cox, London, England, 1952, p. 675.
- (8) "British Pharmacopoeia," The Pharmaceutical Press, London, England, 1958, p. 884.
- (9) Block, R. J., LeStrange, R., and Zweig, G., "Paper Chromatography, A Laboratory Manual," Academic Press Inc., New York, N. Y., 1952, p. 136.

# Investigation of *Machilus macrantha* Nees. II\*

## Pharmacological Action and Chemical Constitution of Machiline

By K. N. GAIND and S. K. BAVEJA

Machiline, isolated as the hydrochloride from the root of *Machilus macrantha* Nees., has been studied for its pharmacological action and chemical characteristics. It has been found to lower blood pressure of experimental animals by a direct depressant effect on the myocardium. It has no antibacterial activity. A number of derivatives have been prepared. Based on the results of (a) microanalysis for elements of the base, its hydrochloride, picrate, acetyl, and benzoyl derivatives, (b) chemical tests for functional groups, (c) microestimation of  $-\text{OCH}_3$  in the base and  $=\text{NCH}_3$  in the methiodide, and (d) infrared and ultraviolet absorption spectra, two structures are proposed for machiline

IN THE PREVIOUS report (1), the isolation of a base "machiline" as a hydrochloride from the root of *Machilus macrantha* Nees was reported. The present investigation was undertaken to study the pharmacological action and chemical constitution of this base.

### METHODS AND RESULTS

**Pharmacological Actions.**—Before undertaking chemical characterization, the isolated hydrochloride was studied for its effect on the cardiovascular system and on smooth muscle and for its antibacterial activity.

**Cardiovascular System.**—Arterial blood pressure was recorded from the common carotid artery in dogs weighing 7–9 Kg, anesthetized with pentobarbital sodium (32 mg/Kg) intravenously. The dose of machiline hydrochloride solution was injected through the cannulated femoral vein. With a dose of 0.1 to 1.0 mg, there was a fall in blood pressure (2 to 11 mm) proportionate to the quantity administered. With larger doses the fall was only slightly greater, the maximum for a dose of 50 mg being 18 mm.

Machiline hydrochloride (10 mg) caused a decrease in the amplitude of contraction of the intact heart in an anesthetized dog with open chest. On the isolated heart (Langendorff's method) (2) of rabbits weighing 1–1.5 Kg, a dose of 0.1 mg caused slowing and irregular rhythm and a dose of 1.0 mg almost stopped the heart by slowing and gradual decrease in the amplitude of contraction. Subsequently the heart recovered gradually in about ten minutes. In the intact heart of a pithed frog, a dose of 0.1 mg caused varying grades of heart block, and a dose of 1.0 mg caused arrest in diastole. Subsequently the heart recovered completely in about five minutes through gradually diminishing grades of heart block.

In perfusion experiments, with 0.1 to 1.0 mg of machiline hydrochloride the flow of frog Ringer's fluid through the peripheral vessels of a frog in-

creased for a few minutes, while the flow of Locke's solution through the coronary vessels of an isolated rabbit's heart decreased for several minutes, but the magnitude of the effects was slight and insignificant. The kidney volume and blood pressure of an anesthetized dog showed simultaneous fall with doses of 1 to 15 mg and this effect was not blocked by previous injection of atropine. These observations suggest that the hypotensive effect of machiline is due to its direct depressant effect on the myocardium.

**Action on Smooth Muscle.**—A dose of 1.0 mg of machiline hydrochloride had a depressant effect on the strips of intestine (Magnus' method) (3) of rabbits weighing 1.0–1.5 Kg, in an isolated organ bath with inner bath of 25 ml capacity.

**Antibacterial Activity.**—In concentrations up to 1 in 500, machiline hydrochloride failed to inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhi* in nutrient broth and of *Mycobacterium tuberculosis* var. *hominis* in the medium suggested by Isao Yamane (4).

**Chemical Characterization of Machiline.**—

**Anal.**—Calcd for  $\text{C}_{16}\text{H}_{17}\text{NO}_3 \cdot \text{HCl}$ : C, 62.41, H, 5.90, N, 4.55, Cl, 11.58. Found: C, 61.46; H, 6.32, N, 4.35, Cl, 11.13.

The picrate from 50 mg of the hydrochloride was precipitated with picric acid in aqueous solution and crystallized from water as yellow crystals (51 mg.) in the form of elongated rectangular plates, melting with decomposition between 205–207°.

**Anal.**—Calcd for  $\text{C}_{16}\text{H}_{17}\text{NO}_3 \cdot \text{C}_6\text{H}_5(\text{OH})(\text{NO}_2)_3$ : C, 52.78, H, 4.03, N, 11.20, Mol wt, 500.2. Found: C, 53.61; H, 4.40; N, 11.47, Mol wt, 465 (Rast method).

The base from 100 mg of the hydrochloride was precipitated with ammonia in aqueous solution and crystallized from 50% alcohol as a microcrystalline white powder (60 mg) melting with decomposition at 210–211°.

**Anal.**—Calcd for  $\text{C}_{16}\text{H}_{17}\text{NO}_3$ : C, 70.81; H, 6.32; N, 5.16; Mol wt, 271.2. Found: C, 70.29; H, 6.60, N, 4.74; Mol wt., 301 (Rast method).

Machiline hydrochloride did not respond to tests with alkaloidal reagents as has already been reported (1). The murexide test for purines was also negative. Heating with dilute as well as concentrated hydrochloric acid did not split the molecule of machiline. It decolorized bromine in aqueous, alcoholic, and glacial acetic acid solutions, and de-

\* Received September 10, 1959, from the Department of Pharmacy, Panjab U-

The authors are  
for his assistance in the pharmacological study and to the Director, National Chemical Laboratory, Poona, for providing microanalytical data.

Verma of  
Amritsar.



colored potassium permanganate in aqueous solution. It did not reduce Fehling's solution, but a silver mirror was formed with ammoniacal silver nitrate. It gave a negative test with Schiff's reagent and also with 2,4-dinitrophenylhydrazine reagent (5). The base neither gave effervescence with, nor dissolved in sodium bicarbonate solution. It dissolved in caustic soda solution and also in ammonia solution, but gave no color with ferric chloride in aqueous or alcoholic solutions. It did not give a green color with gallic acid reagent (6) or a red color with the phloroglucinol-sulfuric acid reagent of Gaebel (7).

*Anal.*—Calcd. for one methoxy in  $C_{16}H_{17}NO_3$ :  $-OCH_3$ , 11.3; Found:  $-OCH_3$ , 7.5.

On heating the base with soda lime, vapors having an ammoniacal odor were evolved and when these were passed into a solution of hydrochloric acid gas in ether a precipitate was formed, which was identified as ammonium chloride. On treating the base with ice-cold nitrous acid, a gas was evolved and a yellow solid precipitated. The latter gave a negative Liebermann test. When the yellow supernatant liquid was made alkaline, no solid separated out. Rimni's (5) and the carbon disulfide reagent (5) tests were also negative.

**Acetylmachiline.**—Machiline hydrochloride (100 mg.), acetic anhydride (1.5 ml.), and pyridine (5 drops) were boiled together under reflux on a sand bath for one hour, cooled, poured into water (80 ml.), set aside overnight, and the precipitate formed was crystallized from 50% alcohol. A crop of white crystals in the form of elongated rectangular plates, m. p. 197–199°, was obtained.

*Anal.*—Calcd. for  $C_{16}H_{15}NO_3 \cdot (CH_3CO)_2$ : C, 67.57; H, 5.96; N, 3.94. Found: C, 66.70; H, 6.00; N, 3.73.

**Benzoylmachiline.**—Benzoylation was effected by the Schotten-Baumann process and the product crystallized from absolute alcohol as elongated rectangular plates, m. p. 214–216°.

*Anal.*—Calcd. for  $C_{16}H_{15}NO_3 \cdot (C_6H_5CO)_2$ : C, 75.12; H, 5.26; N, 2.92. Found: C, 76.20; H, 5.54; N, 2.88.

**Machiline Methiodide.**—Machiline (100 mg.) in methanol (5 ml.) and methyl iodide (0.5 ml.) were warmed together under reflux for one hour. The volatile components of the reaction mixture were volatilized off and the residue dried to constant weight at 120° (154 mg.) was crystallized from dry acetone in a desiccator containing ether. The crystallized compound, after drying at 120°, melted between 184–187°.

*Anal.*—Calcd. for  $C_{16}H_{17}NO_3 \cdot CH_3I$ :  $-CH_3$  (attached to N), 3.63. Found:  $-CH_3$  (attached to N), 3.51.

**Infrared Spectrum.**—The infrared spectrum of machiline (Fig. 1) was taken in a Nujol mull on a single beam Perkin-Elmer spectrophotometer making necessary corrections for interferences due to atmospheric water and carbon dioxide.<sup>1</sup> The brief analysis (Table I) of the infrared record indicates the groups responsible for some of the absorption bands; the assignments were done on an empirical basis.

**Ultraviolet Spectra.**—The ultraviolet spectra (Fig. 2) were taken on a Hilger Uvispek photo-

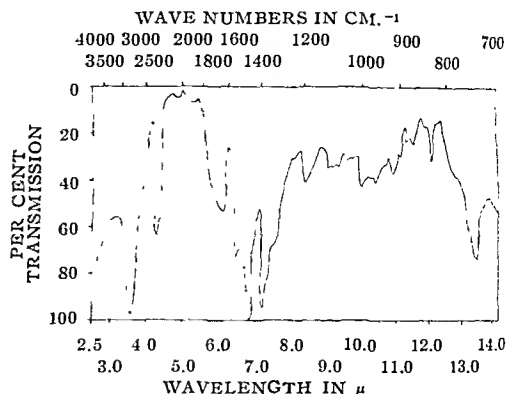


Fig. 1.—Infrared spectrum of machiline.

TABLE I.—EMPIRICAL ASSIGNMENT OF GROUPS RESPONSIBLE FOR ABSORPTION BANDS IN THE INFRARED SPECTRUM OF MACHILINE

Absorption Band Peak, cm. <sup>-1</sup>	Assigned Grouping
3175	CH <sub>2</sub>
2381	≡N (these should be weak bands but were found as strong bands due to faulty compensation)
2105	
2000	
1835	
1600	C <sub>6</sub> H <sub>6</sub> , C=C, C=N in N heterocyclic (?)
1504	C <sub>6</sub> H <sub>6</sub> substituted, C <sub>6</sub> H <sub>5</sub> N (?)
1399	C <sub>6</sub> H <sub>5</sub> OH, $-C-CH_3$ (?)
1333	C <sub>6</sub> H <sub>5</sub> -N=, C <sub>6</sub> H <sub>5</sub> OH, $-OH$
1250	C <sub>6</sub> H <sub>5</sub> OR
1212	1:2, 1:4, 1:2:4 substituted C <sub>6</sub> H <sub>6</sub>
1093	
1058	
1020	
976	N (heterocyclic), $(CH_3)_2C$ (?), $C=CH-$ (?)
813	
719	N (heterocyclic)

electric spectrophotometer equipped with a fused silica prism.<sup>2</sup> Both machiline and its hydrochloride showed approximately the same absorption maxima in the ultraviolet region and no absorption in the visible region of light. The base showed absorption maxima at 226 and 285 mμ in absolute alcohol; its hydrochloride at 200, 224, and 282 mμ; and there was no inflexion near 315 mμ even in a more concentrated solution. The maxima were comparable to those of naphthalene and quinoline except for the lack of inflexion. The inflexion could be absent if (a) a quinoline structure were present as its tetrahydro form or (b) a naphthalene structure were present as 1-phenylnaphthalene (or 2'-hydroxy-1-phenylnaphthalene or 2'-methoxy-1-phenylnaphthalene, etc.). The latter structure would make it rather difficult to fit in nitrogen present as an amine group which in such cases would shift the maxima too much in the acidic condition, whereas not much shifting was observed in the curve of base hydro-

<sup>1</sup> The spectrum was obtained through the courtesy of Dr. M. L. Dhar of the Central Drug Research Institute, Lucknow.

<sup>2</sup> The spectra were prepared in the laboratory of the Royal Institute of Technology, Glasgow, through the courtesy of Dr. H. C. Mital.

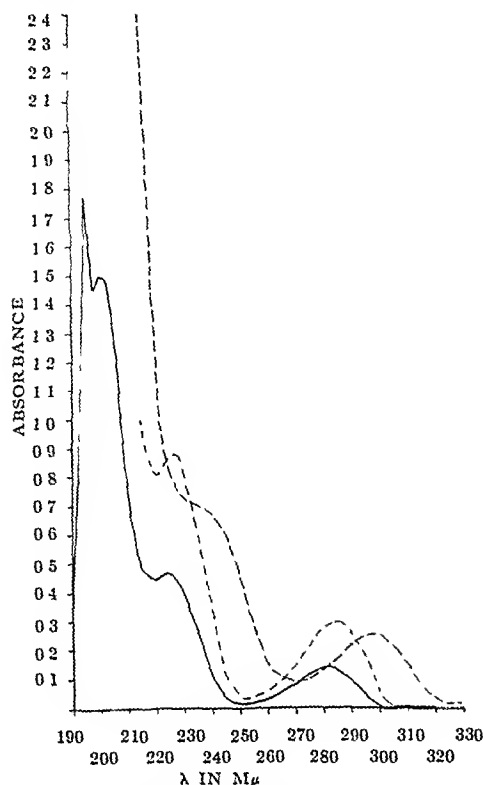


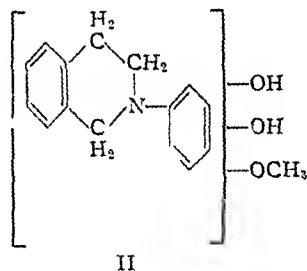
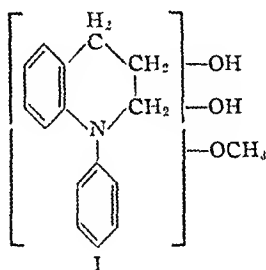
Fig 2—Ultraviolet spectra of machiline—  
 — machiline hydrochloride in water 0.0009818%;  
 ---- machiline hydrochloride in decinormal sodium hydroxide 0.0009818%; -.-.-.- machiline base in ethanol 0.0014374%

chloride. Taking the quinoline structure, its tetrahydro form would not show marked shift in maxima and is in agreement with the observed maxima of base hydrochloride. In alkaline solution the spectrum of machiline hydrochloride was markedly influenced (by the alkali) above 230  $m\mu$ , indicating that nitrogen function is near a benzene ring, i.e., there is a quinoline rather than an isoquinoline system or else a phenyl group is attached to nitrogen in the isoquinoline form.

## DISCUSSION

The microanalytical data of the base and its derivatives show that the molecular formula of machiline is  $C_{16}H_{17}NO_4$  and it has two OH groups and one  $OCH_3$  group. The solubility of the base in alkali hydroxides and the infrared spectrum indicate that at least one of the OH groups is phenolic though it may be in a somewhat hindered position. It does not give the characteristic test for phenols with ferric chloride solution but many other phenolic and enolic compounds behave similarly (7) and, therefore, phenolic OH cannot be considered absent. Chemical tests, infrared spectrum, formation of a

quaternary ammonium compound with methyl iodide accompanied with increase in weight of 100 mg. of the base to 154 mg of the methiodide, and microanalytical data for  $—CH_3$  (attached to N) in the methiodide, all point to the tertiary nature of the nitrogen function. Further the infrared spectrum indicates the nitrogen function to be in a heterocyclic system. The ultraviolet spectra corroborate this finding; specifically, there is a tetrahydroquinoline (or tetrahydroisoquinoline with a phenyl group attached to N) structure. This, together with  $OCH_3$  and OH groups, accounts for  $C_{16}H_{17}NO_4$  leaving  $C_6H_5$  which evidently constitutes a phenyl group and must be attached to a nitrogen function to account for the tertiary nature of the latter in the tetrahydroquinoline (or tetrahydroisoquinoline) nucleus. This conclusion is further strengthened by the maxima at  $1,333\text{ cm}^{-1}$  of the infrared spectrum. On this basis the likely structures for machiline are those represented by I and II



The investigated alkaloids of laurels are known (8) to possess an aporphine ring structure, which has tetrahydroisoquinoline nucleus and on this analogy, structure II may be considered more likely for machiline

## REFERENCES

- (1) Gaind, K. N., and Baveja, S. K., *THIS JOURNAL*, **49**, 659(1960).
- (2) Burn, J. H., "Practical Pharmacology," Blackwell Scientific Publications, Oxford, 1952, p. 25.
- (3) Gaddum, J. H., "Pharmacology," 4th ed., Oxford University Press, London, 1953, p. 213.
- (4) Isao, Y., *J. Bacteriol.*, **73**, 172(1957).
- (5) Vogel, A. I., "Textbook of Practical Organic Chemistry," 1st ed., Longmans, Green and Co., London, 1948, p. 917.
- (6) Nadkarny-Kothare, "Organic Chemistry for Advanced Students," Part I, The Popular Book Depot, Bombay, 1952, p. 383.
- (7) Gaebel, G. O., *Arch. Pharm.*, **248**, 225(1910).
- (8) Henry, T. A., "The Plant Alkaloids," 4th ed., J. A. Churchill Ltd., London, 1949, p. 319.

# The Selective Determination of Isopropamide Iodide, A Low-Molecular Weight Quaternary Ammonium Compound\*

by RALPH S. SANTORO

Available indicator extraction methods were inadequate for the determination of isopropamide iodide in the presence of amine bases. The substance was complexed with methyl orange in pH 10.2 buffer and extracted into chloroform. Re-extraction of the color into 1 *N* hydrochloric acid and its spectrophotometric determination gave quantitative results. Calibration curves for other short chain quaternary amines were determined and compared to a known solution of methyl orange. Agreement with the theoretical value was obtained down to tetra-*n*-propyl ammonium iodide. Application of the method to the determination of isopropamide iodide in a pharmaceutical preparation gave results ranging from 98.7 to 101.3 per cent of the theoretical amount present.

IN AN INVESTIGATION to determine a low-molecular weight quaternary amine, isopropamide iodide,<sup>1</sup> in the presence of amine bases, it became evident that existing indicator extraction methods were inadequate because of poor reproducibility and sensitivity.

Analytical procedures for quaternary ammonium compounds are well represented in the literature. Auerbach (1, 2) determined cetyl pyridinium chloride and other high-molecular weight quaternary amines using bromophenol blue. Cochin and Woods (3) assayed tetraethylammonium with bromocresol purple, extracting the complex from pH 7.0 buffer with chloroform. Ballard, Isaacs, and Scott (4) attempted to elucidate the principles of complex extraction of amines and determined the calibration curves for large chain quaternary compounds using bromothymol blue. They were unsuccessful in determining low-molecular weight quaternaries due to poor complexing and extraction. Other workers have determined large chain surfactants by indicator displacement methods (5, 6, 7).

Similar methods for amine bases are also well documented, usually differing from quaternary methods by the pH at which the complex is extracted. Brodie and Udenfriend (8) determined cinchona alkaloids with methyl orange in acid medium and indicated its application to other amine bases. Keller and Ellenbogen (9) adapted this method for the determination of *d*-amphetamine. Adaptions using different indicators, pH conditions, and solvents have also been described (10-13).

It was found that bromophenol blue, bromo-

thymol blue, and bromocresol purple extractions were incomplete, with a corresponding lack of reproducibility in the results. Alterations in pH and the use of different solvents did not improve the extraction or the reproducibility. Below pH 7.0, the amines complexed and interfered with the determination. No reaction was shown in the direct photometric methods by indicator displacement.

This paper describes an indicator extraction method to determine isopropamide iodide selectively and its use in the determination of certain other low-molecular weight quaternary amines.

## EXPERIMENTAL

**Reagents.**—*Chloroform*, reagent grade. *Hydrochloric acid solutions*, 1.0 *N* and 0.1 *N*. *Buffer solution pH 10.2*, 87 Gm. of dipotassium hydrogen phosphate, reagent anhydrous, and 42 Gm. of sodium carbonate, reagent anhydrous, were dissolved in 2 L. of distilled water. *Methyl orange buffer solution*, 1 L. of 10.2 buffer solution was saturated with methyl orange, reagent grade, by adding 2 Gm. and shaking vigorously. The solution was filtered through Whatman No. 1 filter paper and extracted with chloroform until the chloroform layer was colorless. *Stock solutions of quaternary ammonium salts*, 50 mg. of salt was dissolved in 500 ml. of distilled water. This solution was diluted 20 to 100 ml. with 0.1 *N* hydrochloric acid. A final concentration of 0.020 mg./ml. of the salt was obtained. *Stock solutions of amine salts*, 10 mg. of each substance was dissolved in 100 ml. of distilled water. The concentration of amine salt was 0.100 mg./ml.

**Apparatus.**—Klett-Summerson photoelectric photometer with micro tubes.

**Preparation of Calibration Curve.**—Into four 250-ml. separatory funnels, 20 ml. of methyl orange buffer solution was added. Into the first three separatory funnels, 1, 2, and 3 ml., respectively, of the final quaternary ammonium salt solution were pipetted. The fourth separatory funnel served as the reagent blank. The solutions were extracted

\* Received August 29, 1958, from Smith Kline and French  
was Darbid, Smith Kline  
moyl-3,3-diphenylpropyl)-  
diisopropylmethyl ammonium iodide.

with four 25-ml. portions of chloroform. The filtrates were collected in 250-ml glass-stoppered Erlenmeyer flasks through a pledget of cotton previously wet with chloroform. After the last extraction, the cotton was washed with a 25-ml. portion of chloroform.

Into each flask was pipetted 10 ml. of 1 *N* hydrochloric acid. The flasks were stoppered and shaken vigorously for about one minute. After the layers separated, at least 2 ml. of the clear supernatant aqueous phase was decanted into a micro-Klett tube. The absorbance was read on a Klett-Summerson photoelectric photometer using a 52 filter. All readings were made against 1 *N* hydrochloric acid. The reagent blank was subtracted from the standard readings and each standard weight in mg. was divided by its reading. The above standard weights were 0.020, 0.040, and 0.060 mg. of the quaternary salt, in that order. The factors obtained were averaged.

**Determination.**—Isopropamide iodide was assayed in the presence of amine bases. The determination was the same as the calibration except that to 0.040 mg. (2 ml.) of the standard isopropamide iodide solution 0.200 mg. (2 ml.) of the amine solution was added. The recovery was calculated as follows:

$$\frac{\text{photometer reading} \times \text{average factor}}{\text{mg. isopropamide iodide added}} \times 100 = \% \text{ recovered}$$

### APPLICATIONS

This method was applied to the determination of isopropamide iodide in the presence of prochlorperazine dimaleate<sup>2</sup> in a pharmaceutical preparation.<sup>3</sup> The sample was ground and shaken with 0.1 *N* hydrochloric acid. A dilution was made such that the final concentration of the quaternary salt was 0.010 mg./ml. The determination was carried out as in the calibration with 5 ml. of the final sample dilution. A sample blank was also run by extracting an additional 5-ml. aliquot from pH 10.2 buffer.

### RESULTS AND DISCUSSION

Theoretical considerations and actual determinations show that the molar composition of the methyl orange complex containing one quaternary amine group is a ratio of 1:1 (14). To determine the completeness of extraction and to compare the calibration curve to a theoretical value, the ratio of the Klett readings divided by the calibration curve concentrations, in terms of the methyl orange ion, were calculated and compared to a known solution of methyl orange in 1 *N* hydrochloric acid (Table I).

Agreement with the theoretical value was obtained beginning with tetra-*n*-propyl ammonium iodide. A marked decrease in absorptivity was noted with the tetrapropyl salt at the 0.060-mg. point, which was consistent throughout the calibrations. With this compound, calibrations and determinations

TABLE I—COMPARISON OF CALIBRATION CURVE RATIOS TO METHYL ORANGE

Substance	mg / 10 ml	Ratio Klett Reading/Concn	Methyl Orange Value, %
Methyl orange	0.030	6.811	..
Tetra- <i>n</i> -propyl ammonium iodide	0.020	6.631	97.4
	0.040	6.532	95.9
	0.060	6.023	88.4
Tetra- <i>n</i> -butyl ammonium iodide	0.020	6.615	97.1
	0.040	6.779	99.5
	0.060	6.685	98.2
Isopropamide iodide	0.020	6.816	100.1
	0.040	6.839	100.4
	0.060	6.783	99.6

TABLE II.—RECOVERY OF 0.040 MG. OF ISOPROPAMIDE IODIDE IN THE PRESENCE OF AMINE BASES

Amine, 0.200 mg	Recovery of Isopropamide Iodide, %		
<i>d</i> -Amphetamine	99.3	99.6	100.3
<i>p</i> (2-Aminopropyl) phenol	99.6	93.3	101.3
Phenylephrine	98.4	95.6	100.8
Ephedrine	99.1	104.5	103.5
Phenoxybenzamine	104.1	100.8	98.4
Morphine	102.4	99.3	100.7
Scopolamine	103.5	97.9	104.3
Codeine	100.9	101.6	100.8

TABLE III.—RECOVERY OF ISOPROPAMIDE IODIDE FROM A PHARMACEUTICAL PREPARATION

Isopropamide Iodide, Theoretical	Prochlorperazine, Theoretical	Isopropamide Iodide, mg Recovered	%
21.30	50.0	21.45	100.7
21.30	50.0	21.27	99.8
21.30	50.0	21.14	99.2
21.30	50.0	21.03	98.7
21.30	50.0	21.58	101.3

would have to be carried out at the 0.040-mg. concentration and below for maximum reproducibility.

No extraction of indicator complex was effected with quaternary amines having a lower molecular weight than tetra-*n*-propyl ammonium iodide. These results indicate that the groups attached to the nitrogen must be at least propyl in size, or have a combined total of twelve carbons.

The recovery of isopropamide iodide in the presence of amine bases (Table II) and from a pharmaceutical preparation containing prochlorperazine (Table III) was quantitative. The precision of the calibration curves used in the determinations is shown in Table IV. The sigma values show a deviation of 2 to 4% depending upon the concentration.

The color formed in the 1 *N* acid layer was found to be extremely stable. Samples and standards read after a two-day period did not materially change (Table V).

<sup>2</sup> Compazine, Smith Kline and French brand of 2-chloro 10 [3-(*N*-methylpiperazinyl) propyl] phenothiazine dimaleate.

<sup>3</sup> Combid Spansule capsule, SKF, a combination of isopropamide iodide and prochlorperazine dimaleate.

TABLE IV—PRECISION OF THE CALIBRATION CURVE

Concn 0.020 mg			Concn 0.040 mg			Concn 0.060 mg		
Reading	Factor $\times 10^3$	Ratio	Reading	Factor $\times 10^3$	Ratio	Reading	Factor $\times 10^3$	Ratio
83	0.241	6.551	167	0.240	6.588	251	0.239	6.602
85	0.235	6.709	160	0.250	6.312	250	0.240	6.575
87	0.230	6.867	176	0.227	6.943	260	0.231	6.839
87	0.230	6.867	176	0.227	6.943	256	0.234	6.733
88	0.227	6.946	170	0.235	6.706	261	0.230	6.865
89	0.225	7.024	179	0.223	7.061	263	0.228	6.917
87	0.230	6.867	179	0.223	7.061	255	0.235	6.707
90	0.222	7.103	177	0.226	6.982	262	0.229	6.891
82	0.244	6.472	168	0.238	6.627	256	0.234	6.733
83	0.241	6.551	175	0.229	6.903	257	0.233	6.760
89	0.225	7.024	180	0.222	7.101	266	0.266	6.996
Average								
86	0.232	6.816	173	0.231	6.839	258	0.233	6.783
Sigma								
3	0.007	0.218	6	0.009	0.253	5	0.004	0.129

TABLE V—STABILITY OF METHYL ORANGE IN 1 N HYDROCHLORIC ACID

Sample No	Original Reading	Reading After Two Days
1	104	104
2	193	194
3	280	280
4	362	364
5	98	97
6	195	188
7	276	278
8	364	362

## SUMMARY

1 An indicator extraction method utilizing methyl orange at a pH of 10.2 and chloroform as solvent is described. This method allows the quantitative determination of certain low-molecular weight quaternary amines with a molecular weight equal to or greater than tetra-*n*-propyl ammonium.

2 This method is rapid, sensitive, and reproducible. Primary, secondary, tertiary amines, and alkaloids do not interfere.

3 Application of the method to the determination of isopropamide iodide in the presence of prochlorperazine dimaleate in a pharmaceutical preparation was quantitative.

## REFERENCES

- (1) Auerbach, M. E., *Ind. Eng. Chem. Anal. Ed.*, **15**, 492 (1943).
- (2) *Ibid.*, **16**, 739 (1944).
- (3) Cochran, J., and Woods, L. L., *J. Pharmacol. Exptl. Therap.*, **101**, 7 (1951).
- (4) Ballard, C. W., Isaacs, J., and Scott, P. G. W., *J. Pharm. and Pharmacol.*, **6**, 971 (1954).
- (5) Colchiman, E. L., *Ind. Eng. Chem. Anal. Ed.*, **19**, 430 (1947).
- (6) Fogh, J., Rasmussen, P. O. H., and Skadhague, K., *Anal. Chem.*, **26**, 392 (1954).
- (7) Perlman, P. L., Johnson, C. B., and Kosinski, R. F., *THIS JOURNAL*, **42**, 483 (1953).
- (8) Broche, B. B., and Udenfriend, S., *J. Biol. Chem.*, **158**, 705 (1945).
- (9) Keller, R. E., and Ellenbogen, W. C., *J. Pharmacol. Exptl. Therap.*, **106**, 77 (1952).
- (10) Gettler, A. O., and Sunshine, I., *Anal. Chem.*, **23**, 779 (1951).
- (11) Lubran, M., *Nature*, **164**, 1135 (1949).
- (12) Marshall, P. B., and Rogers, E. W., *Biochem. J.*, **39**, 258 (1945).
- (13) Oberst, F. W., *J. Pharmacol. Exptl. Therap.*, **79**, 10 (1943).
- (14) Ballard, C. W., Isaacs, J., and Scott, P. G. W., *J. Pharm. and Pharmacol.*, **6**, 980 (1954).

# The Biological Action of Cellular Depressants and Stimulants V\*

## The Effect of Phenylurethane on Cellular Synthesis by *Tetrahymena pyriformis* GL

By WALTER SINGER† and JOHN J. EILER

When *tetrahymena* were synchronized by heat treatment in the presence of a  $6 \times 10^{-4}M$  concentration of phenylurethane, the drug inhibited the increase in cell size and reduced the amount of protein synthesis normally found to occur during the period of heat treatment. Deoxyribonucleic acid (DNA) synthesis was not significantly inhibited. When this concentration of phenylurethane was added to *tetrahymena* cultures shortly after the end of the heat treatment, the drugged cells were not different from the controls in cell size, in protein content, or in DNA content at the onset of first synchronous division.

AS A PART of a general study of narcotic action we have continued our investigation into the effect of phenylurethane on parameters related to synchronous growth and cell division of *Tetrahymena pyriformis* GL. Previously we have reported that the drug delays the onset of synchronous divisions and reduces the magnitude of the maximum division indexes (1). This paper presents evidence that phenylurethane inhibits the synthetic processes giving rise to the increases in cell size and cell protein which take place during the period of temperature cycling. Deoxyribonucleic acid (DNA) synthesis does not appear to be inhibited.

### METHODS

Details of culture technique, cell counting, and synchronization procedure have been given (1). Eight temperature shocks of  $34^\circ$  alternated with  $28.2^\circ$  levels were used to synchronize the cells (2). The protozoocrit method of Elliott (3) was used to measure cell volumes. Each protozoocrit was calibrated with inereury to the 0.01 mark. Five- or ten-milliliter suspensions of cells killed by the addition of 0.25 or 0.5 ml of formalin were centrifuged at 2,500 r p m for fifteen minutes, using the No. 303 metal shield in the International centrifuge No. 2 with the No. 240 head. The volume of packed cells was divided by the total cell number to obtain the volume of the average cell. Tests of the reproducibility using eight 10-ml. suspensions pipetted from the same log phase culture showed a standard error of the mean volume of about  $\pm 1.7\%$ .

For DNA and for some protein assays, cells were killed by holding the suspensions for thirty seconds in a  $90^\circ$  water bath. After centrifugation and washing with 0.24% NaCl solution, DNA was

extracted by two fifteen-minute hydrolyses with 5% trichloroacetic acid (TCA) at  $90^\circ$ . Extraction of DNA by this procedure was found to be as complete as by the more involved Schneider method (4). Hydrolysates were combined and DNA content determined by the Dische indole method (5), as modified by Cerriotti (6) and Keek (7). Salmon sperm DNA (California Foundation for Biochemical Research) was used as the standard. The TCA insoluble residue of cellular material from the DNA-extracted *tetrahymena* was dissolved in 3% NaOH and assayed for protein content by the biuret method of Robinson and Hogden (8), using crystalline plasma albumin (Armour) as the standard. In some experiments, this protein assay was carried out using the TCA-insoluble material remaining after formaldehyde-killed *tetrahymena* had been carried through the Schneider extraction method for DNA (4).

### EXPERIMENTAL AND DISCUSSION

The effect of phenylurethane was studied under two general conditions, (a) when the drug was added to log phase cultures just before heating the bath water up to the  $34^\circ$  level for the first temperature shock and (b) when the drug was added to synchronized cultures approximately fifteen minutes after the last (eighth)  $34^\circ$  level. In either case, 0.05 ml. of a stock solution of the drug in 95% ethanol was added, aseptically, to 25 ml. of culture to yield a final concentration of  $6 \times 10^{-4} M$  with respect to phenylurethane. At the same time, suitable controls were provided by adding 0.05 ml. of 95% ethanol to equivalent cultures.

The experiments with the drug added before heat treatment were designed to observe the effect of phenylurethane upon the period of synchronous growth of the cell during the heat treatment and upon the subsequent first lag period. To provide basic reference points, the cell volume, DNA, protein, and cell count were always determined on samples taken from the log phase cultures immediately prior to temperature cycling. In various experiments, these same determinations were made for drugged and control cells (a) at about fifteen minutes after the end of the last  $34^\circ$  level and (b)

\* Received October 30, 1959, from the School of Pharmacy, University of California Medical Center, San Francisco 22.

This paper is adapted from the prize-winning manuscript submitted by Walter Singer in the 1958 Lunsford Richardson Pharmacy Awards competition.

† Fellow of the American Foundation for Pharmaceutical Education, 1955-1956, and Smith Kline and French Fellow, 1956.

at the onset of first synchronous division. Technical factors precluded measuring all of these parameters at both stages within any one experiment. Successful synchronization could be carried out only if the experimental cultures were maintained with a reasonably large surface-volume ratio. Accordingly, for synchronization only 25 ml. of culture was added to a 300-ml. Erlenmeyer flask. To provide sufficient cells for all the samples required, it was necessary to use at least six flasks in each experiment; three for control cells, three for drugged cells. It was then necessary to pool two of the flasks from each type of cell in order to have a sample of the size to permit the determination of cell DNA, protein, volume, and number. The remaining flask of each type of cell was used to follow the synchronous divisions. With these conditions and using a proteose-peptone and yeast extract medium, an excellent first synchronous division was always seen; the maximum division index averaged 0.73 (1). Second synchronous divisions were sometimes seen to occur, third divisions were not.

**The Effects of  $6 \times 10^{-4}$  M Phenylurethane Added Before Heat Treatment.**—Phenylurethane was found to modify the morphological changes observed to occur in tetrahymena during the heat treatment. After the eighth level at  $34^\circ$ , nondrugged tetrahymena were clearly enlarged relative to log phase cells. Most cells were distorted from the typical pyriform shape, there being an overall swelling which was particularly pronounced at the posterior two-thirds of the body. The cell apices were distinctly rounded rather than pointed. Protuberances, apparently randomly distributed about the cell body, could be seen. During the first lag period, the protuberances seemed to disappear and the cells began to look more nearly typical in shape. After the first synchronous division, the cells, though enlarged, had the general appearance of log phase cells.

The cells which were heat-treated in the presence of phenylurethane appeared enlarged at the end of the heat treatment, but were visibly smaller than the nondrugged cells at the same stage. The drugged cells were more nearly elliptical and, though somewhat swollen, showed less distortion than the controls. Cells with protuberances were rarely seen. No differences were noted in transparency or in motility of drugged cells compared to controls.

Tables I and II present data to show the extent of the increases which were found to occur in volume, protein content, and DNA content per average control and drugged cell during the heat treatment and during the first lag period. For the controls, there was an almost threefold increase in average cell volume by the end of temperature cycling (Table I). This agrees with previously reported increases in size during eyseling (2, 9). When temperature-cycled in the presence of  $6 \times 10^{-4}$  M phenylurethane, tetrahymena increased in cell volume only about twofold (Table I). The average drugged cell volume was 76% (Table I) of that of the control at the end of the heat treatment.

It has been shown that tetrahymena which have been heat-treated in the presence of phenylurethane will begin first synchronous division about twenty minutes later than the control cells (1). The

average size of the drugged cells at seventy-eight minutes after heat treatment was compared to that of nondrugged cells at fifty-nine minutes after (Table I). At these respective times, each suspension had just entered into the phase of first synchronous division. The average drugged cell volume was found to be only 81% of that of the control. The values for the averaged cell volumes were derived by calculation from the total packed volume of a known number of cells. It is, therefore, possible that the apparent lower volume of the average drugged cell was the result of a change in cell size distribution such that there was an increased proportion of abnormally small cells in the drugged culture. On the other hand, there could have been an overall proportional decrease in cell sizes without a notable change in distribution of sizes. It was considered important to distinguish between these two alternatives in order to permit a more adequate correlation of the volume data with the results of the study of the drug effect on the synchronous divisions. Consequently, the effect of phenylurethane on the distribution of cell sizes was investigated.

Scherbaum (9) has studied cell size distribution in normal and in synchronized cell populations. His method required calculation of cell volume from measured lengths and widths of a number of individual cells by use of the formula for volume of a prolate spheroid ( $V = \frac{4}{3} \pi ab^2$ ). Bonner and Eden (10) point out that there is no one "correct" or "logical" way of plotting data for frequency distribution of cell sizes and recommend that such data simply be presented in the clearest and most direct manner. Accordingly, in this investigation the frequency distributions of cell lengths and widths were determined, but volumes were not calculated. Photomicrographs were placed in a microfilm reader and the lengths and widths of the magnified images of the cells were measured to the nearest 0.5 mm. The final magnification (275X) was such that 1.0 mm. of the image equalled  $3.63 \mu$  of the original cell. All of the cells in each photograph were measured, this amounting to about twenty cells in each instance. Five pictures of nondrugged cells just beginning to enter first synchronous division were examined; from the same experiment, five pictures of drugged cells likewise at the onset of first synchronous division were selected.

The data for cell lengths (Fig. 1) show the distribution of cell sizes within the drugged culture to be similar to that within the control. The phenylurethane brought about a shift of the entire range of cell sizes in the direction of smaller cells rather than the formation of a group of abnormally small cells within an otherwise normal cell size distribution. This is evidence that, when ready to begin the first synchronous division, the average drugged cell must have been smaller than the average nondrugged cell. Thus the delay in onset of first synchronous division was not brought about by a requirement for the drugged cells to grow up to the size of the control cells in order to divide.

The data in Table I show that during the temperature cycling the protein content per average nondrugged cell increased about threefold. Thus, protein synthesis and increase in cell volume kept

TABLE I—THE EFFECT OF PHENYLURETHANE ON CELL VOLUME, PROTEIN, AND DNA CONTENT OF *Tetrahymena pyriformis* GL<sup>a</sup>

Per Average Cell	Log Phase	After Heat Treatment Controls	After Heat Treatment Drugged	At Onset of 1st Synchron Div Controls <sup>b</sup>	At Onset of 1st Synchron Div Drugged <sup>c</sup>
Volume ( $\mu^3 \times 10^3$ ) <sup>d</sup>	14 2 $\pm$ 0 6	38 9 $\pm$ 2 1	29 1 $\pm$ 0 9		
Ratio to log phase	1 00	2 74	2 05		
Per cent of control			76		
Volume ( $\mu^3 \times 10^3$ ) <sup>e</sup>	16 8 $\pm$ 0 3			45 6 $\pm$ 0 2	36 7 $\pm$ 2 5
Ratio to log phase	1 00			2 71	2 18
Per cent of control					81
Protein (mcg $\times 10^{-3}$ ) <sup>f</sup>	1 44 $\pm$ 0 06	4 44 $\pm$ 0 17	3 41 $\pm$ 0 13		
Ratio to log phase	1 00	3 09	2 37		
Per cent of control			77		
Protein (mcg $\times 10^{-3}$ ) <sup>g</sup>	1 85 $\pm$ 0 19			5 37 $\pm$ 0 34	4 30 $\pm$ 0 11
Ratio to log phase	1 00			2 96	2 38
Per cent of control					81
DNA (mcg. $\times 10^{-5}$ ) <sup>d</sup>	1 69 $\pm$ 0 14	3 11 $\pm$ 0 18	2 79 $\pm$ 0 08		
Ratio to log phase	1 00	1 84	1 65		
Per cent of control			90		
DNA (mcg $\times 10^{-5}$ ) <sup>e</sup>				2 86 $\pm$ 0 49	2 79 $\pm$ 0 10
Ratio to log phase				1 69	1 65
Per cent of control					98

<sup>a</sup>  $6 \times 10^{-4}$  M phenylurethane added to culture medium before heat treatment <sup>b</sup> Measured at fifty nine minutes after the end of the heat treatment <sup>c</sup> Measured at seventy eight minutes after the end of the heat treatment <sup>d</sup> Average of 6 experiments <sup>e</sup> Average of 2 experiments <sup>f</sup> Average of 9 experiments

The standard errors were calculated from the formula

$$S.E. = \pm \sqrt{\frac{\sum(X - \bar{X})^2}{n(n-1)}}$$

TABLE II—THE EFFECT OF PHENYLURETHANE ON CELL VOLUME, PROTEIN, AND DNA CONTENT OF *Tetrahymena pyriformis* GL<sup>a</sup>

Per Average Cell	Log Phase	After Heat Treatment	At Onset of 1st Synchron Div Controls <sup>b</sup>	At Onset of 1st Synchron Div Drugged <sup>c</sup>
Volume ( $\mu^3 \times 10^3$ ) <sup>d</sup>	15 9 $\times$ 0 2	36 2 $\pm$ 1 4	43 5 $\pm$ 2 7 <sup>e</sup>	40 3 $\pm$ 1 8 <sup>e</sup>
Ratio to log phase	1 00	2 28	2 74	2 53
Per cent of control				93
Protein (mcg $\times 10^{-3}$ ) <sup>d</sup>	1 75 $\pm$ 0 07	4 39 $\pm$ 0 23	4 40 $\pm$ 0 26	4 67 $\pm$ 0 31
Ratio to log phase	1 00	2 53	2 53	2 68
Per cent of control				106
DNA (mcg $\times 10^{-5}$ ) <sup>f</sup>	1 94 $\pm$ 0 01		4 67 $\pm$ 0 12	4 58 $\pm$ 0 03
Ratio to log phase	1 00		2 44	2 40
Per cent of control				99

<sup>a</sup>  $6 \times 10^{-4}$  M phenylurethane added to culture medium after heat treatment <sup>b</sup> Measured at approximately sixty minutes after the end of the heat treatment <sup>c</sup> Measured at approximately sixty two minutes after the end of the heat treatment <sup>d</sup> Average of 4 experiments <sup>e</sup> The average cell volume at onset of synchronous division was statistically different from that after heat treatment according to the *t* test ( $p < 0.05$ ) <sup>f</sup> Average of 2 experiments

The standard errors were calculated from the formula

$$S.E. = \pm \sqrt{\frac{\sum(X - \bar{X})^2}{n(n-1)}}$$

pace during the heat treatment Phenylurethane inhibited this protein synthesis to the same extent as it did the volume increase After cycling was completed, the average protein content of the drugged cell, though increased more than twofold, averaged only 77% of that of the control cell at the same stage (Table I) At the onset of synchronous division, the drugged cells had not yet synthesized as much protein as had the nondrugged (Table I) This correlates with the smaller drugged cell volume observed at the onset of synchronous division

The deoxyribonucleic acid (DNA) content of control cells was found nearly to double during the heat treatment (Table I) Comparable results are in the literature (11, 12) A fourfold increase in DNA content per cell also has been reported (13) When the drugged cultures were temperature-cycled, the average DNA per cell increased

by a factor of 1 65 Application of the *t*-test (14) shows that the difference between the DNA content of drugged and control cells was not statistically significant ( $p > 0.05$ ) At the time of the onset of first synchronous division, there was likewise no significant difference between the DNA content of drugged and control cells (Table I) Apparently, phenylurethane had less effect upon DNA synthesis than upon synthesis of protein and increase in cell size during temperature cycling

The Effects of  $6 \times 10^{-4}$  M Phenylurethane Added After Heat Treatment.—When phenylurethane was added at approximately fifteen minutes after the end of the heat treatment, i e., to already synchronized cells, the drug showed no significant effect upon cell volume, protein content, or DNA content during the subsequent first lag period. Data in Table II show each of these attributes to



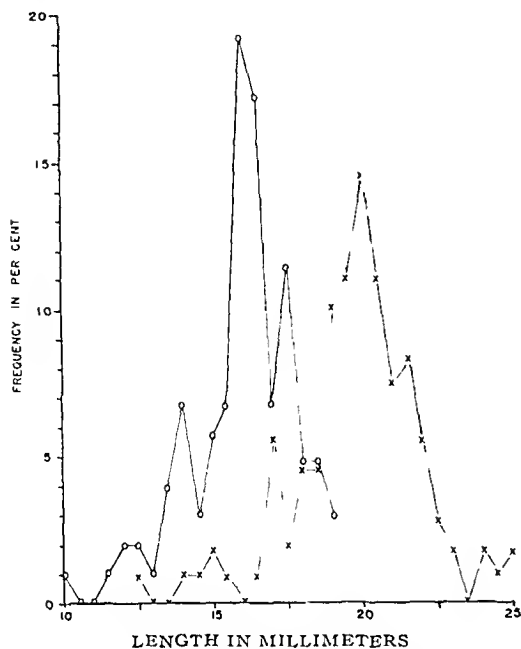


Fig. 1—The effect of phenylurethane on the distribution of cell lengths;  $6 \times 10^{-4} M$  phenylurethane added before heat treatment. X, controls; O, druged. The lengths presented in the figure are those of the projected cell and can be multiplied by 3.3 to give actual cell lengths in microns.

be equivalent for druged and control cells at the onset of first synchronous division. It should be pointed out that under these experimental conditions, druged cells entered into first synchronous division from five to ten minutes later than did the controls, and the magnitude of maximum division index was decreased by about 12% (1). Also, second synchronous division of druged cells was even more delayed (by about twenty minutes) and its maximum division index was reduced to about 50% of the control value of 0.28 (1).

These data also show that under our experimental conditions there was a 20% increase in cell size, but no synthesis of protein during the first lag period. Christensson (15) has found that protein synthesis stops and that cell protein content may even fall during the first lag period. Sherbaum (11) has reported dry weight to increase by 25% during this interval and that "the mean cell size increases slightly before the first division" (9). In another work (16), he and co-workers found an increase of 8-22% in cell volume to occur between the end of the heat treatment and the onset of synchronous division and that the changes in cell density (dry weight divided by volume) which did occur showed no characteristic pattern. Our data show increase in volume and protein content to parallel during heat treatment and suggest that the cell volume increase during the first lag period could be due to water uptake not associated with the synthesis of protein. Whether growth does or does not take place between the end of the heat treatment and the onset of first synchronous division may be related to the number of exposures to

the 34° level. Some workers (9, 15) have used eight temperature shocks as we have; a series of seven shocks (11, 16) has also been used. Furthermore, variations in the degree of nutritional enrichment of the several culture media employed in these investigations may have established different ultimate limitations upon the rate and the total extent of growth possible both during the heat treatment and the first lag period.

In view of the frequently proposed relationship between DNA and cell division, it is interesting that we were unable to show phenylurethane inhibition of DNA synthesis. Druged cells were found to be delayed in entrance into first synchronous division even though averaging approximately the same DNA content as nondruged cells (Tables I and II). Our results are perhaps related to those of Ducoff (17) who has reported that X-ray inhibition of the onset of synchronous division occurred when tetrahymena were irradiated after the heat treatment was completed. The fact that the onset of division was delayed even though the tetrahymena had an abnormally large DNA content at the time of irradiation was considered evidence against the hypothesis that X-ray irradiation inhibits cell division by preventing DNA synthesis.

It would appear that cell size *per se* is not a major controlling factor in the initiation of synchronous cell division. The average druged cell may be smaller than the average nondruged cell at the time of division (when heat-treated in contact with phenylurethane), or it may be the same size as the nondruged cell and yet divide later (when phenylurethane is added to already synchronized cells). Prescott has reported that initiation of cell division does not depend on the attainment of a fixed critical size for asynchronously growing tetrahymena (18) or for amoebae (19), although amoeba cell size may influence the length of the cell cycle (20). Similar evidence indicates that the total cellular protein content does not dictate when synchronous division shall occur. As with DNA, the possible importance of a quantitatively insignificant but very specific protein cannot be dismissed from consideration. It should also be observed that the notable inhibition by phenylurethane of the marked growth and protein synthesis which occurred during the heat treatment was accompanied by pronounced effects upon the subsequent first synchronous division. On the other hand, when the drug was added at the start of the first lag period during which little growth or protein synthesis was found to occur, the effects upon the first synchronous division were quantitatively much lessened.

## SUMMARY

1. When tetrahymena were synchronized by heat treatment, the average cell volume and cell protein were increased threefold and the DNA twofold during the period of temperature cycling.

2. When tetrahymena were synchronized by heat treatment in the presence of  $6 \times 10^{-4} M$  phenylurethane, the increases in cell volume and

protein occurred to a lesser degree. After temperature cycling, the drugged cells averaged 76% of the volume and 77% of the protein of the nondrugged cells. DNA content of drugged cells was not significantly lower than that of nondrugged. At the onset of their division (eighty minutes after temperature cycling) the drugged cells were smaller, had a lower protein content, and the same DNA content when compared to the nondrugged cells at the onset of their division (sixty minutes after temperature cycling). The phenylurethane did not alter the relative distribution of cell sizes, but did shift the normal range of cell sizes in the direction of smaller cells.

3. When  $6 \times 10^{-4} M$  phenylurethane was added to already synchronized tetrahymena shortly after the end of the temperature cycling, the drugged cells were not significantly different from the controls in volume, protein, or DNA at the onset of first synchronous division (about six minutes later for drugged cells than for controls).

4. The implications of the phenylurethane inhibition of cellular synthesis are discussed in relation to its effects upon synchronous divisions.

## REFERENCES

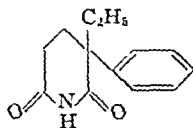
- (1) Singer, W., Lee, K. H., and Eiler, J. J., *This Journal*, **49**, 90(1960)
- (2) Scherbaum, O., and Zeuthen, E., *Exptl. Cell Research*, **6**, 221(1954)
- (3) Elliott, A. M., *Trans. Am. Microscop. Soc.*, **58**, 97(1939)
- (4) Schneider, W. C., *J. Biol. Chem.*, **161**, 293(1945)
- (5) Dische, Z., *Mikrochemie*, **8**, 4(1930)
- (6) Cerriotti, G., *J. Biol. Chem.*, **198**, 297(1952)
- (7) Keck, K., *Arch. Biochem. Biophys.*, **63**, 446(1956)
- (8) Robinson, H. W., and Hogden, C. G., *J. Biol. Chem.*, **135**, 707(1940)
- (9) Scherbaum, O., *Exptl. Cell Research*, **11**, 464(1954)
- (10) Bonner, J. T., and Eden, M., *ibid.*, **11**, 265(1956)
- (11) Scherbaum, O., *ibid.*, **13**, 24(1957)
- (12) Iverson, R. M., and Giese, A. C., *ibid.*, **13**, 213(1957)
- (13) Zeuthen, E., and Scherbaum, O., *Colston Papers*, **7**, 141(1954)
- (14) Lacey, O. L., "Statistical Methods in Experimental," The MacMillan Co., New York, N. Y., 1953
- (15) Christensson, E., *Acta Physiol. Scand.*, **45**, 339(1959)
- (16) Scherbaum, O. H., Louderback, A. L., and Jahn, T. L., *Exptl. Cell Research*, **18**, 150(1959)
- (17) Ducoff, H. S., *ibid.*, **11**, 218(1956)
- (18) Prescott, D. M., *J. Protozool.*, **4**, 252(1957)
- (19) Prescott, D. M., *Exptl. Cell Research*, **9**, 328(1955)
- (20) Prescott, D. M., *ibid.*, **11**, 86(1956)

# A Quantitative Fluorometric Reaction for Glutethimide\*

By R. P. HAYCOCK, P. B. SHETH, and W. J. MADER

A fluorometric procedure is described for the determination of glutethimide (2-ethyl-2-phenylglutarimide) in tablet formulations which is based on the fluorogen resulting from its reaction with concentrated sulfuric acid containing formaldehyde. The fluorescent intensity is a straight line function of concentration over a wide range. The excitation and fluorescent spectra were determined with a spectrophotofluorometer as an aid in studying the method.

GLUTETHIMIDE<sup>1</sup> is a central nervous system depressant, hypnotic, and sedative. Its structural formula is



An examination of the literature shows a scarcity of methods for its determination.

Using *p*-nitrobenzeneazoresorcinol as the indicator and pyridine or ethylenediamine as the solvent, glutethimide may be titrated as a

monobasic acid with sodium methoxide (1). Direct titration of glutethimide in powdered tablet samples is not possible if the tablet excipients are acidic unless it is used in conjunction with other techniques to isolate glutethimide from interfering substances. Goldbaum, Williams, and Koppanyi (2) recognized that glutethimide in strong alkaline solutions possesses a strong absorption maximum at 235 mμ with an absorptivity (1%, 1 cm) of 880, whereas in methanol, glutethimide exhibits a very weak absorption as shown in curve A, Fig. 1. The occurrence of strong absorptivity in alkaline solutions is presumably related to resonance of the molecule permitted by ionization of the nitrogen-bound hydrogen atom (3). However, the rapid hydrolysis of gluteth-

\* Received April 7, 1960, from the Research Department, Ciba Pharmaceutical Products, Inc., Summit, N. J.

<sup>1</sup> Ciba's trade name for glutethimide is Doriden.

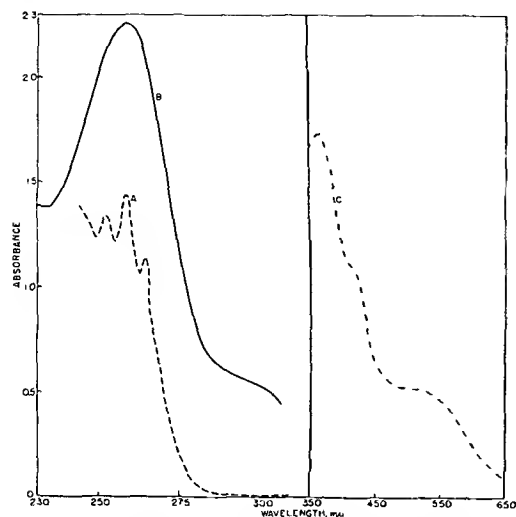


Fig. 1.—Absorption curves of glutethimide. *A*, Glutethimide in methanol 0.4 mg./ml.; *B*, formaldehyde-sulfuric acid fluorogen of glutethimide 0.02 mg./ml.; *C*, formaldehyde-sulfuric acid fluorogen of glutethimide 0.2 mg./ml.

imide in strong alkaline solution is not conducive to its determination in pharmaceutical preparations. Sheppard (4) and associates utilized a method based on the formation of a colored complex between ferric ion and the hydroxamate resulting from the reaction of glutethimide with alkaline hydroxylamine for the determination of the drug in urine. The presence of lactose and anions which complex with ferric ion or salts which form precipitates with ferric ion interfere with the method. Notwithstanding the adaptability of these methods to a particular problem, they present difficulties and ambiguities in the determination of glutethimide in certain tablet formulations.

The authors have observed that glutethimide when heated with concentrated sulfuric acid containing formaldehyde gives a reddish coloration with an intense blue fluorescence. The transformation of glutethimide into a fluorogen with formaldehyde-sulfuric acid reagent is the basis of the method of analysis to be described. Optimum conditions for the development of the fluorescences have been determined, and the applicability of the reaction to related compounds is noted.

### EXPERIMENTAL

The fluorescent properties of formaldehyde-sulfuric acid treated glutethimide were studied using the Amineo-Bowman spectrophotofluorometer in order to facilitate the selection of filters for the quantitative measurements. The instrument was calibrated against quinine by the method of Sprince

and Rowley (5). The excitation and fluorescent spectra which characterize the fluorogen were obtained using a Moseley model No. 3 flat bed XY recorder as shown in Fig. 2. Measurements were made at room temperature in a quartz cell having a 1-cm. light path using  $1/16$  inch defining slit (band pass = 6 mμ). An RCA 1P21 photomultiplier tube was used to detect the emission. The excitation spectrum is represented by a plot of the exciting wavelength against the intensity of the fluorescent light. The fluorescent spectrum represents the relative intensity of the fluorescent light over the range of 200 to 600 mμ. Excitation maxima were observed at 280 and 365 mμ, and the corresponding maximum fluorescent emission occurred at 450 mμ. The peaks checked within 5–10 mμ on repeated tests.

Absorbance measurements of the fluorogen were made with a Cary recording spectrophotometer as shown in curves *B* and *C*, Fig. 1. A maximum at 257 mμ in the ultraviolet region and a maximum at 365 mμ in the near visible region are indicated. The wavelength of greatest excitation seems to coincide with the strongest absorption band (365 mμ) nearest the fluorescent spectrum band.

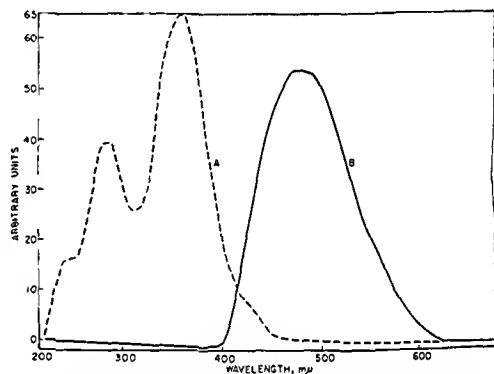


Fig. 2.—Excitation and fluorescent spectra of formaldehyde-sulfuric acid fluorogen of glutethimide. *A*, Excitation scan—fluorescence held at 450 mμ; *B*, fluorescent scan—excitation held at 380 mμ.

### PROPOSED QUANTITATIVE PROCEDURE

**Reagents.**—Unless otherwise indicated, all reagents are reagent grade. Formaldehyde-sulfuric acid reagent: dilute 10 ml. of 37% formaldehyde to 100 ml. with concentrated sulfuric acid with cooling. Prepare fresh daily; diluted sulfuric acid, 50%; dilute 100 ml. of water with 100 ml. of sulfuric acid cautiously with cooling; reference standard glutethimide: use crystalline glutethimide assayed by phase solubility (6), solubility in 50% aqueous methanol is 15.1 mg./Gm.; standard glutethimide solution: prepare standards containing 0.1, 0.3, 0.5, 0.7, and 1.0 mg./ml. in methanol.

**Apparatus.**—Use a photofluorometer such as the Coleman photofluorometer model 12C with broad band Corning 5970 filter (PC-6) to isolate the exciting radiation (365 mμ) and a combination Corning 3389–4308 filter (PC-1) which transmits 450 mμ to isolate the fluorescent light emitted.

**Procedure.**—Weigh a counted number of not less than 20 tablets, and reduce them to a fine powder. Transfer an accurately weighed portion of the pow-

der, equivalent to 50 mg of glutethimide, to a 100-ml. volumetric flask. Dilute to volume with methanol and shake. Centrifuge a portion of the mixture and use the clear supernatant liquid for fluorescent development (concn. = 0.5 mg./ml.).

Pipet 1 ml. of each standard solution of glutethimide, 1 ml. of the sample preparation, and 1 ml. of methanol into separate 50-ml. volumetric flasks. Add 5 ml. of formaldehyde-sulfuric acid reagent, and heat in the oven for twenty minutes at 100° with occasional swirling of the contents of each flask. Cool the flasks to room temperature, dilute to volume with diluted sulfuric acid, adjust the temperature to 25°, and shake well. Allow the flasks to stand for fifteen minutes to permit air bubbles to escape. Adjust the photofluorometer to an arbitrary point, 75, with the highest standard and at zero with the blank using the standard cell supplied with the instrument. Determine the instrument readings on the other standards and the sample preparation. A plot of fluorescent intensity vs concentration, as shown in Fig. 3, is linear. Ascertain the concentration of sample preparation from the graph. In routine analyses it is unnecessary to run several concentrations of standard. Use a standard containing 0.5 mg./ml. and calculate the quantity of glutethimide in mg. in the sample by the formula  $(A-B)/(S-B) \times 50$ , where  $A$  and  $S$  are the fluorescent readings of the sample and standard, respectively, and  $B$  is the blank.

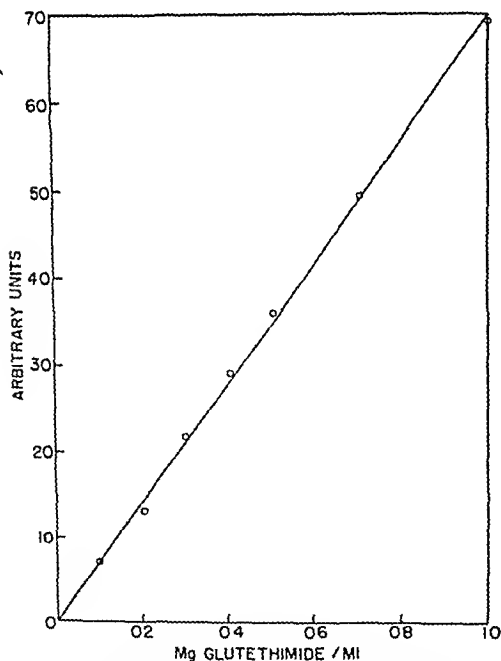


Fig. 3.—Plot of fluorescence of final solution against initial concentration of glutethimide.

### DISCUSSION

In order to determine the optimum reaction time for the formation of the fluorogen, the fluorescence of the final solution as a function of heating was investigated. From the curve shown in Fig. 4, it can be seen that at 100° a constant maximum fluores-

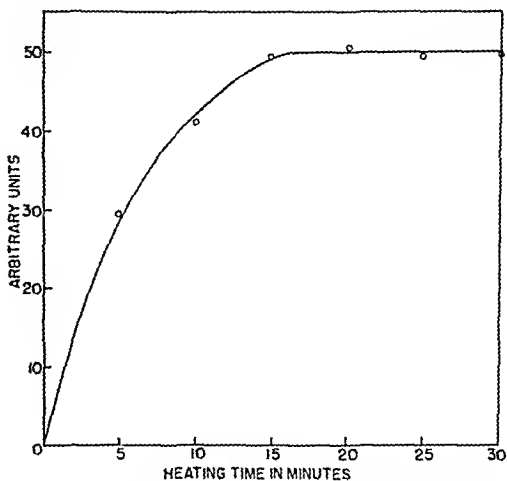


Fig. 4.—Plot of fluorescence of final solution against heating time of reaction. Initial concentration of glutethimide was 0.5 mg./ml.

cence is obtained after fifteen minutes. On this basis twenty minutes was selected for the assay procedure. Temperatures below 100° were unsatisfactory, and at room temperature the fluorogen was not formed.

The behavior of glutethimide toward concentrated sulfuric acid was determined in advance of the addition of formaldehyde, but no fluorescence was detected indicating that the formaldehyde was an essential factor in the reaction. Figure 5 illustrates the effect of the concentration of formaldehyde on the fluorescent intensity. The fluorescence of the solution remained constant and stable for a reagent containing 6 to 20 ml. of formaldehyde for each 100 ml. of sulfuric acid. For convenience, 10 ml. of formaldehyde was used in the reagent for the proposed analytical method. Subsequent experimental

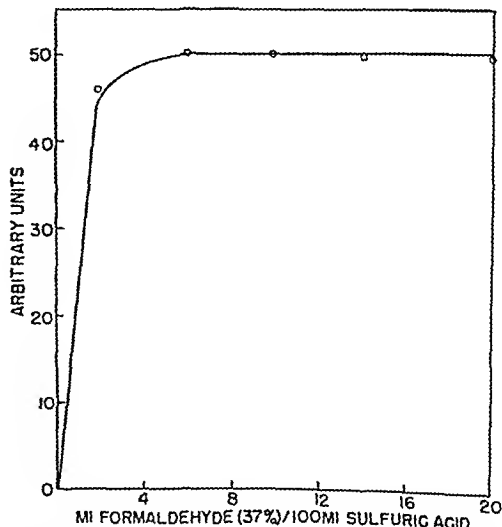


Fig. 5.—Plot of fluorescence of final solution against concentration of formaldehyde. The initial concentration of glutethimide was 0.5 mg./ml.

TABLE I—COMPARISON OF FLUORESCENT AND COLORIMETRIC PROCEDURES

Sample	Description and Declaration	Glutethimide, % of Declaration	
		Fluorescent	Colorimetric
1	Tablets, 125 mg	100.6	100.0
2	Tablets, 125 mg.	100.5	100.6
3	Tablets, 250 mg	100.3	99.6
4	Tablets, 500 mg	98.5	98.4
5	Tablets, 250 mg with reserpine, 0.2 mg	101.5	100.4
6	Tablets, 125 mg with reserpine, 0.1 mg	99.5	100.8
7	Tablets, 125 mg with reserpine, 0.1 mg	103.3	102.4
8	Tablets, 125 mg with methylphenidate, <sup>a</sup> 5 mg	101.5	99.9
9	Tablets, 125 mg with methylphenidate, 10 mg	100.7	100.0
10	Capsules, 125 mg	104.0	107.0

<sup>a</sup> Ciba's trade name for methylphenidate is Ritalin.

data indicated that trioxymethylene could be substituted for the formaldehyde solution.

The addition of water to the fluorogen formed on heating glutethimide with formaldehyde-sulfuric acid reagent destroys the fluorescence. However, the final solution may be diluted to volume with diluted sulfuric acid in concentrations greater than 40% without adversely affecting the intensity of the fluorescence. In the assay procedure 50% sulfuric acid was more suitable and was used in the proposed method.

The authors have analyzed several lots of tablets containing glutethimide and combinations with other drugs by both the hydroxamic acid colorimetric procedure and the proposed fluorometric technique. The colorimetric procedure required preliminary extraction with chloroform to separate glutethimide from lactose. The summary of these analyses, as shown in Table I, indicates that the two methods yield concordant assay values. Common tablet excipients such as starch, powdered sugar, lactose, stearic acid, magnesium stearate, talcum, and magnesium aluminum stearate do not interfere with the

TABLE II—FLUORESCENCE OF GLUTETHIMIDE AND SOME RELATED COMPOUNDS

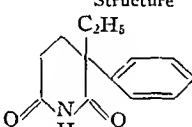
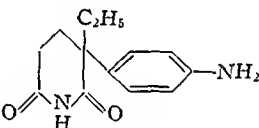
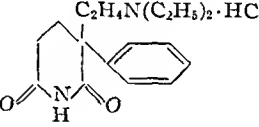
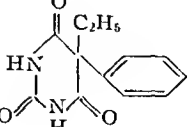
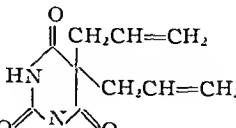
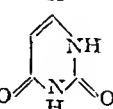

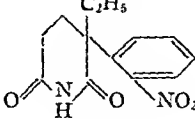
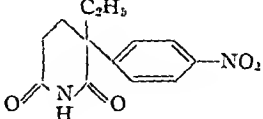
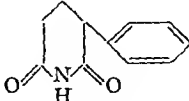
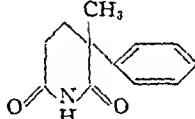
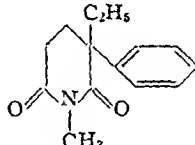
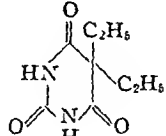
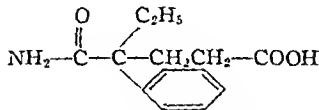
Compound	Structure	Relative Fluorescent Intensity
Glutethimide		100
Aminoglutethimide <sup>a</sup>		Nil
Phcnglutarimide		23
Phcnobarbital		73
Diallylbarbituric acid <sup>c</sup>		23
Uracil		Nil
Benzene		Nil (colored)

TABLE II (continued)

Compound	Structure	Relative Fluorescent Intensity
2-Ethyl-2-( <i>o</i> -nitrophenyl)-glutarimide		Nil
2-Ethyl-2-( <i>p</i> -nitrophenyl)-glutarimide		Nil
2-Phenylglutarimide		78
2-Methyl-2-phenylglutarimide		96
2-Ethyl-N-methyl-2-phenylglutarimide		100
Diethylbarbituric acid		Nil
4-Ethyl-4-phenylglutaramic acid		10

<sup>a</sup> Ciba's trade name for aminoglutarimide is Elipten  
trade name for diallylbarbituric acid is Dial

<sup>b</sup> Ciba's trade name for phenylglutarimide is Aturban

<sup>c</sup> Ciba's

fluorescent technique. The totality of results on several commercial lots performed in triplicate showed a precision of  $\pm 2\%$ .

In order to determine the selectivity of the reaction and the effect of various moieties on the molecule, a number of related compounds were studied, as summarized in Table II. The proposed assay is applicable to certain structurally related derivatives containing an unsubstituted phenyl group. Except for diallylbarbituric acid, the reaction does not occur with compounds lacking a phenyl group or compounds containing a substituted phenyl moiety. The order of magnitude of fluorescence depends upon certain groups in the basic molecule which may retard or prevent the fluorogenic reaction. The procedure is applicable for the determination of glutethimide in the presence of its degradation product, 4-ethyl-4-phenylglutaramic acid, since the latter yields only one-tenth the fluorescence of glutethimide. Although the chemistry of the reac-

tion of glutethimide with formaldehyde-sulfuric acid reaction is obscure, it seems likely that concentrated sulfuric acid brings about a condensation of the compound with formaldehyde and then oxidizes the resulting compound to a fluorescent compound. Phosphoric acid which behaves like sulfuric acid with respect to dehydration but, in contrast, never acts as an oxidant does not form the fluorescent compound in the presence of formaldehyde.

## REFERENCES

- (1) Ciba Research Reports, 1956
- (2) Goldbaum, M. W., Williams, M., and Koppanyi, T. *Federation Proc.* 16, 300(1957)
- (3) Klotz, I. M., and Askounis, T. *J. Am. Chem. Soc.* 69, 801(1947)
- (4) Sheppard, H., D'Asaro, B. S., and Plummer, A. J. *This Journal*, 45, 681(1956)
- (5) Sprince, H., and Rowley, G. R., *Science*, 125, 25(1957)
- (6) Mader, W. J., "Organic Analysis," Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1954, pp. 254-275

# Adaptation of the Chromotropic Acid Method to the Assay of Spans\*

By LACEY GATEWOOD, Jr., and HORACE D. GRAHAM

The chromotropic acid method for the determination of formaldehyde has been adapted to the direct and quantitative determination of the Span surfactants. The proposed method involves saponification of the particular Span to yield sorbitol and the particular fatty acid. The sorbitol portion is then oxidized by periodate to yield formaldehyde which is coupled with chromotropic acid to yield a wine color with an absorption maximum at 570  $m\mu$ . This procedure has been found to be rapid and simple with a high degree of sensitivity.

THE SPANS are nonionic surfactants produced through the esterification of various fatty acids and sorbitan, the dehydrated form of sorbitol, a hexahydroxy alcohol. They are widely used in pharmaceutical products and their reactions influence the efficacy and stability of pharmaceutical formulations. Their many applications, including some recent ones (3), allegedly involve an interaction between the surfactant and specific ingredients. The concentration of these surfactants used, in most cases, is very low. The exact nature of the mechanisms whereby they exert their influence can be ascertained, therefore, only if rapid, precise, and sensitive methods for their determination are readily available. To date, no rapid colorimetric method for assay of this class of surfactants has been reported (6). Therefore, these studies were initiated to develop a rapid colorimetric method for their quantitative determination exploiting the chromotropic acid method for formaldehyde (4) which has been modified and adapted to, among other things, the estimation of mannitol and sorbitol (2, 8).

If subjected to alkali saponification, the Spans, in aqueous solution, should yield sorbitol and the particular fatty acid. The sorbitol portion should, therefore, be estimable by the chromotropic acid method to give a direct measure of the concentration of the particular Span. This premise was experimentally verified and constitutes the basis of this paper.

## EXPERIMENTAL

**Materials and Reagents.**—Span 20, sorbitan monolaurate, lot 333; Span 40, sorbitan monopalmitate, lot 8068c; Span 60, sorbitan monostearate, lot 7615c; all obtained from the Atlas Powder Co., Wilmington, Del. Potassium hydroxide, 0.5 *M*, U. S. P., Fisher Scientific Co. Periodic acid reagent, 0.0075 *M*. Stannous chloride reagent, 0.0035 *M*. Chromotropic acid reagent, disodium salt, practical grade, Eastman Kodak Co., Rochester 3, N. Y. These reagents were prepared according to West and Rapoport (8).

**Equipment.**—Coleman universal spectrophotometer, model 14; 20-ml. Pyrex-stoppered test tubes.

## GENERAL PROCEDURE

**Saponification Step.**—In all preliminary studies, Span 20 only was used. One gram was weighed into a 250-ml. Erlenmeyer flask. To this 50 ml. of 0.5 *M* potassium hydroxide were added. The flask was placed in a boiling water bath for twenty minutes and after cooling the contents were quantitatively transferred to a 1,000-ml. volumetric flask and made up to volume with distilled water. When necessary, further dilutions were made with distilled water to obtain solutions for the subsequent oxidation and color development steps.

**Oxidation of the Sorbitol Produced Through Saponification and Color Development With Chromotropic Acid.**—Solutions containing the equivalents of 300, 400, and 500  $\text{mcg./ml.}$  of the original Span were made up from the saponified sample by appropriate dilution with distilled water. One milliliter of each of these solutions was pipetted into duplicate 20-ml. glass-stoppered Pyrex test tubes. A control (in duplicate) containing distilled water instead of the saponified Span was included. Periodic acid reagent, 0.5 ml., was added to each tube and the contents were thoroughly mixed. The tubes were allowed to stand for eight minutes at room temperature ( $28 \pm 1^\circ$ ). At the end of this period, 0.5 ml. of the stannous chloride reagent was added to each tube, the contents again mixed well, and the tubes placed in a cold water bath. Six milliliters of the chromotropic acid reagent and 2 ml. of distilled water were added to each tube and the tubes immersed in a boiling water bath for thirty minutes. After removal and cooling, the color intensity was measured at 570  $m\mu$ . Confirmation of the wavelength of maximum absorption was obtained by measuring the absorbance over the wavelength range of 400–700  $m\mu$  and, as shown in Fig. 1, is at 570  $m\mu$ , which is the same as that for formaldehyde, as found by Bricker and Johnson (1), and for sorbitol, as previously established (8) and reproduced here. Span 40 and Span 60 gave absorption spectra similar to that of Span 20.

\* Received November 27, 1959, from the George Washington Carver Foundation, Tuskegee Institute, Alabama.

The authors wish to acknowledge gifts of the various samples of Spans used from the Atlas Powder Co., Wilmington, Del.

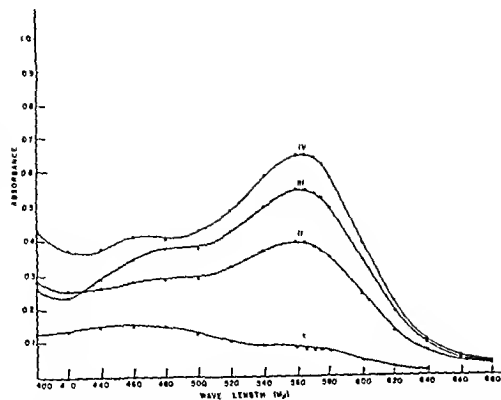


Fig 1—Absorption spectra of chromotropic acid complexes of formaldehyde and the periodate oxidation products of sorbitol and Span 20. I, blank; II, Span 20; III, sorbitol, IV formaldehyde

The concentration of a particular Span in an unknown can be determined in exactly the same way and calculated from a standard curve for the Span. If the amount of formaldehyde produced is used as the basis of calculation, then this can be determined from a standard curve for the formaldehyde-chromotropic acid reaction, established according to the general procedure but omitting the saponification step.

**Influence of Saponification Time.**—Duplicate solutions containing 400 mcg of Span 20 were saponified for times indicated in Table I and the intensity of the color developed measured at 570 mμ.

The results indicate that the difference in per cent transmission for saponification times of ten to thirty minutes fell well within the range of experimental error. At and above forty minutes, color development was more intense but no constancy was observed. In view of the constant response over the lapse of ten to thirty minutes, a saponification period of thirty minutes was chosen.

TABLE I—EFFECT OF SAPONIFICATION TIME ON COLOR INTENSITY

Saponification Time, min	Transmission, %	Selected Time, min
10	27.8	30
20	27.5	
30	27.0	
40	25.2	
50	23.2	

RELATIONSHIP BETWEEN COLOR INTENSITY AND CONCENTRATION OF SPAN

After the optimal saponification time was established, aliquots of the saponified Span corresponding to 0, 100, 200, 300, 400, and 500 mcg of the original Span were placed in duplicate ground-glass-stoppered test tubes. The samples were oxidized for eight minutes, the color developed according to the general procedure, and its intensity measured at 570 mμ. Preliminary experiments had shown that for color development an oxidation time of eight

minutes, 0.5 ml. of the periodate solution containing  $3.75 \times 10^{-6}$  moles of potassium periodate,  $1.75 \times 10^{-6}$  moles of stannous chloride,  $11.25 \times 10^{-6}$  moles of chromotropic acid, and a heating time of thirty minutes were optimal. These limits for this system correspond well with those established by Bricker and Johnson (1) for formaldehyde.

When the logarithm of the per cent transmission as recorded in Table II is plotted as a function of the

TABLE II—RELATIONSHIP BETWEEN CONCENTRATION OF SPAN 20 AND THE INTENSITY OF THE COLOR DEVELOPED

Concentration of Span 20, mcg / 10 ml	Transmission, %		Average
	Expt I	Expt II	
0	87.0	85	86.0
100	63.9	62	63.0
200	46.9	44.8	45.8
300	33.4	32.8	33.1
400	24.5	24.7	24.6
500	19.0	18.0	18.5

concentration of added Span, a linear relationship results. This indicates that a quantitative relationship exists and that Beer's law is being obeyed.

**Recovery of Span 20 from Mixtures Containing Anionic Surfactants.**—Since pharmaceuticals may contain anionic surfactants, it was thought of interest to study recovery from a mixture containing some common forms. For this, 1.0 Gm of Span 20, 0.5 Gm of sodium lauryl sulfate, 1.0 ml of sodium tetradecyl sulfate, and 0.5 Gm of sodium lauryl sulfonate were mixed with 50 ml of 0.5 M potassium hydroxide in a 125-ml Erlenmeyer flask. The mixture was placed in a boiling water bath for thirty minutes. Aliquots of the mixture calculated to contain 10, 20, and 40 mcg. of the added Span 20 were oxidized and the color developed as in previous experiments.

The results summarized in Table III show that high recovery of Span 20 was achieved in the presence of the anionic detergents used.

TABLE III.—RECOVERY OF SPAN 20 FROM MIXTURES OF ANIONIC SURFACTANTS

Amount Added, mcg	Span 20 Recovered, mcg	Recovered, %
10	10	100.0
20	18.9	94.5
40	38.8	97.0

DISCUSSION

Experimental evidence has been obtained to demonstrate that the chromotropic acid method for the determination of formaldehyde can be adapted to the direct assay of the "Span" class of surfactants. Preliminary alkali saponification of the Span is necessary in order to release the sorbitan moiety for subsequent oxidation to formaldehyde. For optimal conditions in the system employed, a saponification time of thirty minutes, an oxidation time of eight minutes, a level of  $3.75 \times 10^{-6}$  moles of potassium periodate,  $1.75 \times 10^{-6}$  moles of stannous chloride,



$11.25 \times 10^{-6}$  moles of chromotropic acid, and a heating time of thirty minutes were selected.

The similarities in the absorption spectra and visual appearance for identically developed colors using formaldehyde, sorbitol, and the Spans confirm that formaldehyde is actually produced after saponification and oxidation. Furthermore, evidence was obtained that the formaldehyde produced from the saponification product of the Span can be determined directly by the chromotropic acid method. Although only three Spans were used, since the other commercially available ones, namely, Span 65 (sorbitan tristearate), Span 80 (sorbitan monooleate), and Span 85 (sorbitan trioleate), all contain the sorbitan moiety, the test can be considered as a class test for the Spans.

Since many formulations are combinations of anionics and nonionics, recovery tests were attempted from mixtures. In the presence of anionics, good recovery was achieved (Table II). Weeks, Ginn, and Baker (7) analyzed for nonionics gravimetrically after ion exchange separation to remove the anionic type. The small quantities of nonionics in most preparations caused them some concern in their otherwise successful approach. The proposed direct spectrophotometric method could supplement the ion-exchange approach. Attempts to make direct assays in the presence of cationic surfactants were unsatisfactory. However, by use of ion exchange resin such cationic surfactants could be removed prior to analysis by the proposed method. Rosen (5) has also outlined a simple, useful procedure for the separation of nonionic surfactants from mixtures with anionics by ion exchange. Polyoxyethylene types of nonionic surfactants will also give a positive test with chromotropic acid. However, as noted by Rosen (5), the oxonium salt of polyoxyethylene compounds can be precipitated by large anions such as cobalthiocyanate, or phosphomolybdate ion. This, then, could offer a possible separation of the Tweens from the Spans after removal of anionic and cationic surfactants by ion exchange resins.

## SUMMARY

The chromotropic acid method for the determination of formaldehyde has been adapted to the quantitative determination of the "Span" surfactants. The particular Span is first saponified to release the sorbitol moiety. Controlled oxidation of the sorbitol by periodate leads to the production of formaldehyde which is then coupled with chromotropic acid to give a wine color, the intensity of which is proportional to the amount of Span present.

Optimum conditions for determination are as follows: a saponification time of thirty minutes, an oxidation time of eight minutes, a level of  $3.75 \times 10^{-6}$  moles of chromotropic acid, and a heating time of thirty minutes for developing the color. These conditions are specified for a total volume of 10 ml. in the system.

Satisfactory recovery was achieved in the presence of anionic surfactants, but recovery in the presence of cationic surfactants was unsatisfactory. For reproducible results it is recommended that all reagents be prepared fresh each day.

## REFERENCES

- (1) Bricker, C. E., and Johnson, H. R., *Ind. Eng. Chem., Anal. Ed.*, **17**, 400(1945).
- (2) Corcoran, A. E., and Page, I. H., *J. Biol. Chem.*, **170**, 165(1947).
- (3) Ferrel, R. E., and Kester, E. B., *Food Technol.*, **13**, 473(1959).
- (4) MacFadyen, D. A., *J. Biol. Chem.*, **158**, 107(1945).
- (5) Rosen, M. J., *Anal. Chem.*, **27**, 787(1955).
- (6) Siggia, S., *J. Am. Oil Chemists Soc.*, **35**, 643(1958).
- (7) Weeks, L. E., Ginn, M. E., and Baker, C. E., *Soap Chem. Specialties*, **1957**, 33.
- (8) West, C. D., and Rapoport, S., *Proc. Soc. Exptl. Biol. Med.*, **70**, 141(1949).

# Notes

## Note on X-ray Crystallographic Data on Some Alkaloids from *Vinca rosea* Linn.\*

By ANN VAN CAMP

A NUMBER of compounds have been obtained from the pantropical plant, *Vinca rosea* Linn. An account of the preparation and characterization of the alkaloids from this plant has been given by Svoboda, Neuss, and Gorman (1).

Crystallographic data on two alkaloids from this plant, reserpine and ajmalicine, have been published by Rose (2, 3). Previously unpublished X-ray crystallographic data on nine alkaloids from this plant are presented here.

### EXPERIMENTAL

Crystals of catharanthine, catharanthinol, and lochnericine suitable for crystallographic work were crystallized from methanol; leurosine was crystallized from acetonitrile, vincalucoblastine etherate, vindoline, and vindolicine were crystallized from ether; vincalucoblastine methanolate was crystallized from methanol, perivine hydrochloride was crystallized from methanol-ether mixture, and virosine was crystallized from chloroform-methanol mixture.

The X-ray powder diffraction data on all samples

except perivine hydrochloride were obtained with chromium radiation and vanadium filter, using a wavelength value of 2.2896 Å. in the calculations. Copper radiation and nickel filter with a wavelength value of 1.5405 Å. were used to obtain the data on perivine hydrochloride. A standard Norelco powder camera, 114.6 mm in diameter, was used.

The single crystal data were obtained using nickel filtered copper radiation and a Weissenberg camera.

### DISCUSSION

Table I gives X-ray single crystal data. Perivine hydrochloride and vindoline give optical biaxial figures. From this fact and from observation of the  $hkl$  intensities, these two alkaloids cannot have higher than orthorhombic symmetry in spite of the apparent equality of the  $a$  and  $b$  cell dimensions.

Table II gives X-ray powder diffraction data. Included in this table are data for three alkaloids, catharanthine, virosine, and vincalucoblastine methanolate, for which no suitable single crystals were available.

TABLE I.—X-RAY SINGLE CRYSTAL DATA

Compound	System	Cell Dimensions	Axial Ratios $a : b : c$	Density	Formula Wt. Per Cell
Catharanthinol $C_{20}H_{24}N_2O$	Ortho	$a = 12.00$ $b = 13.52$ $c = 10.48$	0.8876 : 1.0 : 0.7751	1.210 Flotation 1.207 X-ray	4
Leurosine $C_{46}H_{58}N_4O_9 \cdot 8H_2O$	Ortho	$a = 20.03$ $b = 26.63$ $c = 9.44$	0.7522 : 1.0 : 0.3545	1.262 Flotation 1.261 X-ray	4
Lochnericine $C_{21}H_{24}N_2O_3$	Ortho.	$a = 13.40$ $b = 13.73$ $c = 9.96$	0.9760 : 1.0 : 0.7254	1.287 Flotation 1.280 X-ray	4
Perivine·HCl $C_{20}H_{24}N_2O_3 \cdot HCl$	Ortho	$a = 15.85$ $b = 15.85$ $c = 7.59$	1.1 : 1.0 : 0.4789	1.303 Flotation 1.307 X-ray	4
Vincalucoblastine etherate $C_{46}H_{58}N_4O_9 \cdot (C_2H_5)_2O$	Mono.	$a = 22.57$ $b = 21.95$ $c = 19.60$ $\beta = 95^\circ 36'$	1.0283 : 1.0 : 0.8930	1.224 Flotation 1.215 X-ray	8
Vindolicine $C_{25}H_{32}N_2O_6$	Mono	$a = 15.94$ $b = 10.61$ $c = 14.03$ $\beta = 95^\circ$	1.5024 : 1.1 : 0.1327	1.288 Flotation 1.284 X-ray	4
Vindoline $C_{25}H_{32}N_2O_6$	Ortho	$a = 15.61$ $b = 15.68$ $c = 9.55$	0.9955 : 1.0 : 0.6090	1.279 Flotation 1.299 X-ray	4

\* Received March 7, 1966.  
Tories, Eli Lilly and Co.  
The author is grateful to

and Dr. M. Gorman for supplying the compounds from which these data were obtained and to Dr. R. R. Pfeiffer for technical advice.

TABLE II—X-RAY POWDER DIFFRACTION DATA

Catheranthine $C_{17}H_{21}N_2O \cdot H_2O$				Vincalucoblastine etherate $C_{46}H_{59}N_4O_9 \cdot (C_2H_5)_2O$					
<i>d</i>	<i>I/I<sub>1</sub></i>		<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>	<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>	
10 51	1 00		4 94	0 40		11 32	0 03	200	4 48 0 07 40 $\bar{3}$ , 430
9 43	0 80		4 85	0 40		9 77	1 00	002	4 35 0 07 204
8 74	1 00		4 71	0 40		9 16 <sup>a</sup>	0 13	201, 211	4 17 0 13 43 $\bar{2}$ , 151
7 12	0 20		4 40	0 10		8 65	0 67	102, 121	4 01 0 27 25 $\bar{1}$
5 65	0 40		1 32	0 40		7 48	0 50	22 $\bar{1}$	3 87 0 03 50 $\bar{3}$
5 49	0 10		3 49	0 20		7 16	0 33	310	3 68 0 07
5 25	0 40					5 97	0 13	31 $\bar{2}$ , 222, 231	3 56 0 13
						5 84	0 13	20 $\bar{3}$	3 43 0 13
						5 50	0 27	040	3 22 0 03
						5 26	0 07	330	3 01 0 07
						5 08	0 07	402	2 89 0 07
						4 82	0 27	24 $\bar{1}$	
						4 59	0 20	20 $\bar{4}$	
Catheranthinol $C_{17}H_{21}N_2O$									
<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>	<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>				
9 16	0 30	110	3 75	0 30	212				
8 17	0 40	011	3 65	0 08	230				
6 78	1 00	020	3 50	0 04	320				
5 24	0 80	002	3 40	0 02	040				
5 08	0 20	121	3 29	0 04	321				
1 90	0 12	012	3 17	0 02					
4 69	0 20	102	3 08	0 08					
4 16	0 30	031	2 96	0 02					
3 90	0 04	131	2 89	0 04					
Leurosine $C_{16}H_{19}N_3O_2 \cdot 8H_2O$									
<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>	<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>				
15 8	0 50	110	5 25	0 30	141				
13 4	0 40	020	4 94	0 05	410				
11 0	0 50	120	4 73	0 10	250				
9 81	0 20	200	4 61	0 30	102				
9 29	0 20	210	4 13	0 05	032				
8 77	0 05	101	4 03	0 05	410				
8 10	1 00	220	3 93	0 10					
7 18	0 50	121	3 76	0 10					
6 59	0 75	211	3 66	0 05					
6 34	0 20	140	3 51	0 05					
6 09	0 20	221	3 38	0 05					
5 47	0 10	041	3 33	0 05					
			3 17	0 05					
Vincalucoblastine methanolate $C_{46}H_{59}N_4O_9 \cdot 2CH_3OH \cdot H_2O$									
<i>d</i>	<i>I/I<sub>1</sub></i>		<i>d</i>	<i>I/I<sub>1</sub></i>					
18 7	0 13		4 58	0 20					
14 2	0 13		4 38	0 20					
10 77	0 13		4 21	0 13					
9 81	0 27		4 00	0 20					
9 32	0 67		3 76	0 03					
8 59	0 67		3 57	0 13					
7 79	0 07		3 44	0 13					
7 44	0 07		3 34	0 03					
7 16	1 00		3 19	0 03					
5 84	0 33		2 99	0 03					
5 64	0 13		2 79	0 03					
5 43	0 27		2 62	0 03					
4 79	0 27								
Lochnericine $C_{17}H_{21}N_2O_1$									
<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>	<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>				
9 66	0 08	110	3 42	0 08	040				
7 95	0 08	101	3 23	0 08	410				
6 88	1 00	020	3 16	0 01	141				
6 08	0 15	120	3 03	0 02	240				
5 62	0 15	021	2 99	0 02	420				
5 16	0 40	121	2 87	0 02	421				
4 83	0 15	220	2 73	0 01	050				
4 59	0 40	030	2 63	0 01	510				
4 32 <sup>a</sup>	0 10	221, 130	2 55	0 01	511				
3 97	0 15	131	2 47	0 01	251				
3 78	0 15	230, 320	2 36	0 01	152				
3 51	0 15	231							
Peruvine hydrochloride $C_{17}H_{21}N_2O_2 \cdot HCl$									
<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>	<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>				
11 32	0 20	110	3 89	0 20	140, 410				
8 03	1 00	020, 200	3 76	0 10	330				
6 96	1 00	101, 011	3 64	0 10	112				
6 34	0 10	111	3 49	0 10	141, 411				
5 62	0 30	220	3 38	0 75	331				
5 09	0 10	130, 310	3 06	0 30	132, 312				
4 56	0 20	221	2 98	0 05	250, 520				
4 42	0 20	230, 320	2 90	0 10	232, 322				
4 22	0 30	131, 311	2 77	0 05	251, 521				
<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>	<i>d</i>	<i>I/I<sub>1</sub></i>	<i>hkl</i>				
14 04	0 60	001	3 50	0 20	004				
9 92	0 40	101	3 29	0 16					
8 02	1 00	200	3 21	0 02	230				
7 14	0 20	201	3 15	0 02					
6 99	1 00	002	3 08	0 04					
6 78	0 20	201	3 02	0 01					
6 31	0 10	210	3 00	0 01					
5 41	0 80	202	2 87	0 02					
5 30	0 08	020	2 70	0 02					
5 14	0 08	301	2 64	0 12					
4 94	0 30	021	2 57	0 04					
4 40	0 02	302, 220	2 55	0 01					
4 25	0 60	013	2 47	0 08					
4 00	0 16	400	2 45	0 02					
3 92	0 16	401	2 33	0 08					
3 80	0 40	312	2 11	0 01					
3 75	0 04	110	2 01	0 01					
3 69	0 20	321							

TABLE II (continued)

Vindoline C <sub>21</sub> H <sub>23</sub> N <sub>3</sub> O <sub>5</sub>			Virosine C <sub>21</sub> H <sub>23</sub> N <sub>3</sub> O <sub>4</sub>			
<i>d</i>	<i>I/I</i> <sub>1</sub>	<i>hkl</i>	<i>d</i>	<i>I/I</i> <sub>1</sub>	<i>d</i>	<i>I/I</i> <sub>1</sub>
11 2	0 33	110	16 83	0 01	3 13	0 01
8 20	1 00	101, 011	8 91	1 00	2 99	0 04
7 86	1 00	200, 020	8 22	0 40	2 91	0 04
7 25	0 50	111	7 79	0 30	2 88	0 04
6 09	0 20	021, 201	6 04	0 01	2 79	0 08
5 66	0 27	121	5 71	0 75	2 66	0 04
5 01	0 27	130, 310	4 89	0 75	2 61	0 04
4 80	0 07	221, 002	4 39	0 10	2 49	0 06
4 55	0 27	301, 102, 012	4 24	0 15	2 35	0 01
4 40	0 50	131, 311, 112	4 07	0 20	2 31	0 02
3 95	0 50	231, 321, 122	3 92	0 04	2 28	0 02
3 81	0 27	140, 410	3 87	0 06	2 22	0 01
3 63	0 20	222	3 72	0 04	2 19	0 01
3 53	0.07	240, 420, 141, 411, 302	3 40	0 15	2 12	0 01
3 45	0 13	331, 132, 213	3 19	0 02		
3 38	0 03					
3 20	0 03					
3 07	0 13					
2 92	0 03					
2 80	0 03					
2 76	0 03					
2 68	0 03					
2 62	0 03					

a Indicates broad line

## REFERENCES

- (1) Svoboda, G. H., Neuss, N., and Gorman, M., *This Journal*, 48, 11, 659(1959) (2) Rose, H. A., *Anal. Chem.*, 26, 1245(1954)  
(3) *Ibid.*, 27, 469(1955)

Note on the Occurrence of Taxine in *Taxus brevifolia*\*

By V. E. TYLER, Jr.

A small amount of an alkaloid isolated from the fresh needles of Pacific yew was shown to be amorphous taxine. No cyanogenetic glycoside could be detected in this plant material. The chemotaxonomic significance of these findings is discussed.

A RECENT SURVEY of the chemotaxonomic significance of the occurrence of alkaloids and cyanogenetic glycosides in the genera *Taxus*, *Cephalotaxus*, and *Torreya* has been conducted by Hegnauer (1). All of the species and varieties of *Taxus* investigated were found to contain alkaloids which were shown chromatographically to be identical with taxine, but these alkaloids were distinctly different from those present in *Cephalotaxus*. Many species and varieties of *Taxus* proved to be cyanogenetic, but all species of *Cephalotaxus* and *Torreya* investigated, as well as some varieties of *Taxus*, were noncyanogenetic.

*Taxus brevifolia* Nutt., the Pacific or western yew, was not one of the species investigated by Hegnauer, although he concluded on the basis of his survey that the complex alkaloidal mix-

ture, taxine, probably occurs in all species of *Taxus*. This statement appeared to be of special interest in view of the report of Jones and Lynn (2) that *T. brevifolia* contains no alkaloid and probably no glycoside. Consequently, it was deemed worth while to re-examine this plant in an attempt to verify the chemotaxonomic significance of taxine in the genus *Taxus*.

## EXPERIMENTAL

**Isolation of an Alkaloid.**—Fresh needles and small twigs of *Taxus brevifolia* collected in October near the summit of Snoqualmie Pass, Washington, were coarsely comminuted by passage through an Abbe mill No 000. A 1,150-Gm. quantity of this plant material was placed in a large beaker and allowed to macerate overnight with 2 L. of 2% hydrochloric acid. It was then divided into portions which were homogenized in a Waring Blendor together with appropriate volumes of an additional 2-L. quantity of 2% hydrochloric acid. The acid extract was separated from the resulting mash by expression through muslin in a hand press. The 3 L. of extract thus obtained was concentrated under reduced pressure in a flash evaporator at 55° to a volume of 800 ml. which was rendered alkaline by the addition of ammonium hydroxide. After extraction in a separatory funnel with three successive 200-ml. portions

\* Received January 22, 1960, from the College of Pharmacy, University of Washington, Seattle 5

of chloroform, the chloroform extracts were combined and evaporated to dryness on a steam bath. This residue was redissolved in 30 ml. of ether which was then shaken out with three 5-ml. portions of hydrochloric acid.

In order to effect a further purification of the alkaloid, the combined hydrochloric acid extracts were rendered alkaline with ammonium hydroxide, re-extracted with three 5-ml. portions of ether, these extracts combined and re-extracted three times with a total of 10 ml. of 2% hydrochloric acid. The alkaloid was precipitated from this solution by the addition of ammonium hydroxide, removed from the chilled solution by filtration, and dried in a desiccator over anhydrous calcium chloride. Yield of the alkaloid was 8.9 mg. or 0.00077% of the starting material, calculated on a fresh weight basis.

**Identification of Amorphous Taxine.**—Since taxine is such a complex mixture of alkaloids which are subject to decomposition, Graf and Bertholdt (3) have concluded that the melting point of the compound has absolutely no relationship to its purity. Likewise, the optical activity displayed by a solution of taxine varies according to the time of harvest of the plant and the botanical source of the compound. Consequently, these classical analytical properties could not be employed in the evaluation of the alkaloid isolated from *T. brevifolia*.

The benzidine and Prussian blue tests for nitrogen (4) yielded negative results when applied to a solution prepared from the products of sodium fusion of small quantities (<3 mg.) of the compound, but the same tests were also negative when applied to similar quantities of known taxine.<sup>1</sup> Subsequently, the isolated product was subjected to a quantitative micro-analytical procedure for nitrogen. The percentages of carbon and hydrogen were also determined, although this was considered as a secondary objective, and the quantity of sample available for this determination was very small (1.811 mg.). This may explain the slight discrepancy in the percentage of carbon found in the sample.

**Anal.**<sup>2</sup>—Calcd. for  $C_{37}H_{51}NO_{10}$ : C, 66.34; H, 7.67; N, 2.09. Found: C, 61.26; H, 7.72; N, 2.15.

The isolated alkaloid also gave positive reactions to a number of colorimetric tests for taxine. A deep red color was formed when it was treated with concentrated sulfuric acid, and when the Salkowski test was applied, a reddish-orange color was formed in the acid layer and a pink color in the chloroform layer (5). When the alkaloid was spotted on filter paper, sprayed with hydrochloric acid, and heated for a few minutes at 100°, it exhibited the characteristic bluish-red color and yellow fluorescence described by Graf (6). When a similar spot on filter paper was sprayed with diluted Wagner's reagent, it gave a citron-yellow color typical of taxine.

Hegnauer (1) has shown that when amorphous taxine is subjected to paper chromatography in a number of common solvent systems it does not separate into its components but is detectable as a single, rather broad spot. Quantities of the isolated alkaloid were spotted on 3 inch X 12 inch sheets of Whatman No. 1 filter paper, not only as the

single compound but also in combination with authentic taxine and with a purified alkaloidal extract freshly prepared from *Taxus baccata* L. These sheets were formed ascendingly for a period of five to six hours with the following solvent systems: *n*-butanol-hydrochloric acid-water (100:10-saturation), *n*-butanol-acetic acid-water (4-1-5), and *n*-propanol-1 *N* ammonia (4-1-1).

The sheets were sprayed with Munier's reagent (7) which gave reddish-orange spots with taxine and with the isolated alkaloid. The  $R_f$  values of the three test substances were identical in each of the three solvent systems employed:

System	Average $R_f$
Butanol-acetic acid-water.....	0.79
Butanol-hydrochloric acid-water....	0.82
Propanol-ammonia.....	0.90

**Test for Cyanogenetic Glycoside.**—The Grignard-Mirande qualitative test for hydrogen cyanide was carried out as described by Hegnauer (8) but employing a double quantity (10 Gm.) of finely chopped, fresh needles of *T. brevifolia*. After incubating for forty-eight hours at 37°, the sodium picrate paper gave no indication of a positive reaction.

## CONCLUSIONS AND DISCUSSION

On the basis of the results of the elemental analyses and various color reactions as well as  $R_f$  values obtained in three different wash liquids, it was concluded that the alkaloid isolated from *Taxus brevifolia* was amorphous taxine. The existence of this alkaloid in this species of *Taxus* in small (0.00077% of fresh weight) but definite amounts is thus confirmed. This is in contradiction to the earlier report of Jones and Lynn (2) which indicated the absence of alkaloids in *T. brevifolia*.

This discrepancy may be explained, at least in part, by the relatively small amount of alkaloid present in this species, and by the fact that Jones and Lynn based their conclusion on the negative character of sodium-fusion tests for nitrogen. In this study it has been shown that such tests do yield negative results when applied to small amounts of amorphous taxine.

The presence of this alkaloid is of considerable interest from the chemotaxonomic viewpoint, since it confirms the reliability of taxine as a genus characteristic and substantiates Hegnauer's postulation to this effect by correcting a misconception present in the older literature. Furthermore, it supports the observation (1) that the smallest quantities of alkaloid are found in those species and varieties of *Taxus* which lack cyanogenetic glycosides, since glycosides of this type were found to be absent in this alkaloid-poor species.

## REFERENCES

- (1) Hegnauer, R., *Pharm. Weekblad*, 94, 241 (1959).
- (2) Jones, I., and Lynn, E. V., *THIS JOURNAL*, 22, 528 (1933).
- (3) Graf, E., and Bertholdt, H., *Pharm. Zentralhalle*, 96, 385 (1957).
- (4) Shriner, R. L., Fuson, R. C., and Curtin, D. Y., "The Systematic Identification of Organic Compounds," 4th ed., John Wiley & Sons, New York, N. Y., 1956, pp. 57-69.
- (5) Cromwell, B. T., "Modern Methods of Plant Analysis," vol. 4, Springer-Verlag, Berlin, Germany, 1955, p. 422.
- (6) Graf, E., "Über die Alkaloide der Bäume," Thesis, Julius-Maximilians-Universität, Würzburg, Germany, 1956, p. 17.
- (7) Block, R. J., Durrum, E. L., and Zweig, G., "A Manual of Paper Chromatography and Paper Electrophoresis," 2nd ed., Academic Press, New York, N. Y., 1958, p. 361.
- (8) Hegnauer, R., *Pharm. Weekblad*, 93, 891 (1958).

<sup>1</sup> A generous sample of amorphous taxine was supplied through the courtesy of Dr. E. Graf, Würzburg, Germany.

<sup>2</sup> Analyses carried out by Drs. G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England.

# Note on the Disappearance of Pharmacologic Activity\*

By JOSEPH V. SWINTOSKY and F. M. STURTEVANT

THE CONCEPT of exponential excretion of drugs is not new (1-4); however, applications of this concept to pharmaceutical practice are of relatively recent vintage (5-8). Although most workers have studied only blood and urine levels, Hill (4) was one of the first to recognize the exponential character of the disappearance of some pharmacologic responses. In the assessment of various pharmaceutical forms of a drug, it is preferable to use performance indexes derived from pharmacologic responses, especially when they have some correlation to clinical therapeutic responses. In addition, such responses reflect the action of the original drug as well as all active metabolites. The present note illustrates the approximate exponential disappearance of pharmacologic response for several drugs and indicates how this knowledge may be applied pharmaceutically.

## EXAMPLES FROM THE LITERATURE

**Pyretogenic Effects.**—Gogerty and Dille (9) measured the pyretogenic effect of *d*-lysergic acid diethylamide (LSD) and of *d*-lysergic acid morpholide (LSM) following i. v. administration into rabbits. The disappearance of fever appeared to be approximately exponential when we replotted their data for 50 mcg./Kg. doses on semilog paper (Fig. 1). By graphical estimation, the half-lives ( $t_{1/2}$ ) for LSM and LSD were approximately 80 and 430 min., and the specific velocity constants ( $k = 0.693/t_{1/2}$ )

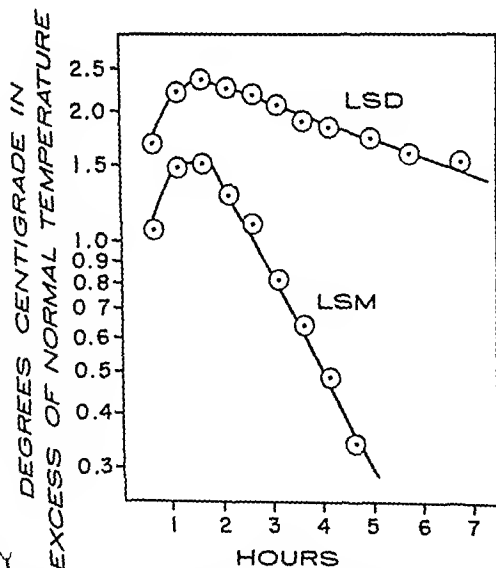


Fig. 1.—Effect on rabbit rectal temperature after intravenous administration of 50 mcg./Kg. of LSM and LSD, respectively. Each curve represents mean values of six rabbits. Data for plots from Gogerty and Dille (9).

\* Received March 30, 1960, from the Smith Kline and French Laboratories, Philadelphia, Pa.  
The authors wish to acknowledge the technical assistance of Mr. Gordon Black.

0.0087<sup>-min.</sup> and 0.0016<sup>-min.</sup>, respectively. It is of interest that the replacement of the diethylamine side chain by the morpholine moiety results in a more rapid elimination of the compound from its receptors. This may also serve to explain the lower peak activity of LSM, although one cannot deny possible differences in affinity and intrinsic activity between LSM and LSD.

**Antisialagogue Effects.**—Carter, *et al.* (10), obtained some limited human data on salivary response following the oral administration of tincture of belladonna. Again, if the data are replotted on semilog paper, the disappearance of the antisialagogue activity appears to follow an exponential course (Fig. 2). The half-life is approximately 1 to 1.5 hours and the velocity constant about 0.6<sup>-hr.</sup>

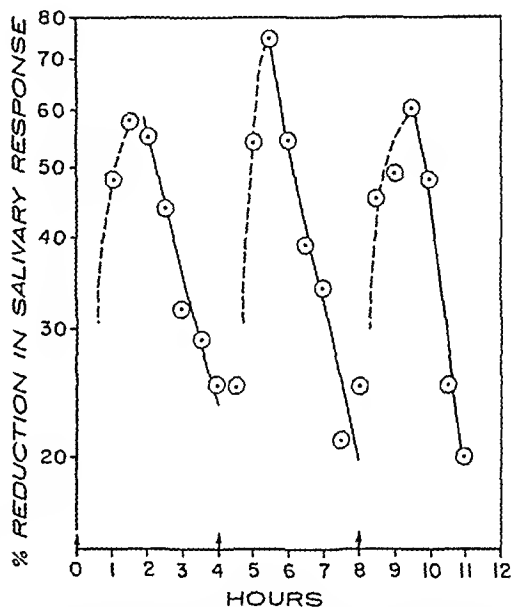


Fig. 2.—Mean response of four experiments in two human subjects given 2.4 ml. tincture of belladonna at zero, four, and eight hours. Data for plots from Carter, *et al.* (10).

## EXPERIMENTAL

Twelve male Dierolf mice were given 5 mg./Kg oral doses of the new anticholinergic agent SKF 5515-I (9-methyl-3-oxo-9-azabicyclo-[3,3,1]-nonan-7-yl-benzilate maleate) (11). At the next five hourly intervals, additional maintenance doses of 1 mg./Kg. orally were given. Measurements of mydriasis were made under constant illumination using an ocular micrometer; these readings were recorded half-hourly in terms of arbitrary units (1 unit  $\approx$  0.2 mm.). The size of the maintenance dose was calculated on the basis of previous experiments (12), in which SKF 5515-I displayed an apparent mydriatic half-life of 2.0 hours in mice; the corresponding velocity constant was 0.35<sup>-hr.</sup> Thus, letting  $W$  = amount of drug in the body which is

activating the mydriatic receptors at a desired level (mg./Kg.),  $W_0$  = extrapolated "initial" amount of drug at  $t = 0$ , which approximates dose if absorption is rapid (mg./Kg.),  $t$  = time (hours), and  $k$  = constant for removal of drug from receptors (hours<sup>-1</sup>)

$$W = W_0 e^{-kt} \quad (\text{Eq. 1})$$

$$W_0 - W = W_0(1 - e^{-kt}) \quad (\text{Eq. 2})$$

To maintain the intensity of the one-hour mydriasis with hourly sustaining doses, we may substitute in the last equation  $W_0 = 5$ ,  $t = 1$ , and  $k = 0.35$ , whereupon  $W_0 - W = 1.5$  mg./Kg. and  $W = 3.5$  mg./Kg. The replenishment dose is then about  $3.5(1 - e^{-0.35}) = 1.0$  mg./Kg. hourly.

### RESULTS AND DISCUSSION

In Fig. 3 it is observed that a fairly constant level of mydriasis was maintained by the replenishment doses of SKF 5515-I. After the last dose, the  $t_{1/2}$  for disappearance of mydriasis was 1.3 hours, which in turn compares favorably with the value of 2 hours determined previously.

In utilizing the preceding equations and calculations, no attempt was made to interpret mechanisms of drug action. Furthermore, the assumption of first-order disappearance of pharmacologic response may be only approximately correct. As has been

shown by Hill and others (4, 13, 14), the drug receptor reaction might be theoretically and experimentally described by a reversible second-order reaction. However, first-order rate equations are more simply handled than those of a higher order. It becomes a practical expedient to interpret data in terms of pseudo first-order reaction rate kinetics when possible.

The utility of the first-order rate kinetic constants for the design of oral sustained action products, for example, may be illustrated. For a sustained mydriatic action at a 3.5 mg./Kg. level of SKF 5515-I, where absorption from the sustained release portion of the dose is zero order, where  $k = 0.35$ , and where the rate of drug elimination,  $dE/dt$ , should ideally equal the rate of absorption,  $dA/dt$ , i. e., where  $dW/dt = (dA/dt) - (dE/dt) = 0$ , one may compute that the constant rate of absorption should be  $dA/dt = kW = 0.35 \times 3.5 = 1.2$  mg./Kg./hour. This is very nearly equal to the replenishment dose required when administered every hour as illustrated in previous calculations.

If, for example, sustained pupil dilation at this level is required for about four hours, the equation for estimating the total dose, initial release, and the maintenance portion of the sustained action dosage unit may be written

$$Wt = W_0 + \int_1^4 k W dt \quad (\text{Eq. 3})$$

(total dose)	(initial release)	(maintenance portion)
	$= W_0 + (k \times W \times 3)$	
	$= 5 + (0.35 \times 3.5 \times 3)$	
	$= 8.6$ mg./Kg.	

The dosage unit, therefore, should contain 5 mg./Kg. for initial release, with 3.6 mg./Kg. available for relatively uniform absorption over about the one to four hour interval. The equation and calculation may assume minor variations depending upon the rate of drug absorption, accuracy with which  $W$  may be determined, and other assumptions.

### REFERENCES

- (1) Arrhenius, S., "Quantitative Laws in Biological Chemistry," G. Bell and Sons, Ltd., London, 1915.
- (2) Storm VanLeeuwen, W., "Grondbegins. d. alg. Pharmacol.," Wolters, Den Haag, 1923.
- (3) Clark, A. J., "The Mode of Action of Drugs on Cells," Edward Arnold & Co., London, 1933.
- (4) Hill, A. V., *J. Physiol. London*, 39, 361 (1909).
- (5) Lapp, C., *Prods. pharm.*, 9, 466 (1954); 11, 87, 162 (1956).
- (6) Swintosky, J. V., Robinson, M. J., Foltz, E. L., and Free, S. M., *THIS JOURNAL*, 46, 399 (1957).
- (7) Swintosky, J. V., Robinson, M. J., and Foltz, E. L., *ibid.*, 46, 403 (1957).
- (8) Swintosky, J. V., Foltz, E. L., Bondi, A., Jr., and Robinson, M. J., *ibid.*, 47, 136 (1958).
- (9) Gogerty, J. H., and Dille, J. M., *J. Pharmacol. Exptl. Therap.*, 120, 340 (1957).
- (10) Carter, K., and Hawkins, D., Private communication.
- (11) Wilton, J. G., and Macko, E., *Federation Proc.*, 18, 458 (1959).
- (12) Sturtevant, F. M., *Proc. Soc. Exptl. Biol. Med.*, 104, 120 (1960).
- (13) Ariens, E. J., van Rossum, J. M., and Simonis, A. M., *Pharmacol. Rev.*, 9, 218 (1957).
- (14) Gehlen, W., *Arch. exptl. Pathol. Pharmacol. Naunyn-Schmiedeberg's*, 171, 541 (1933).

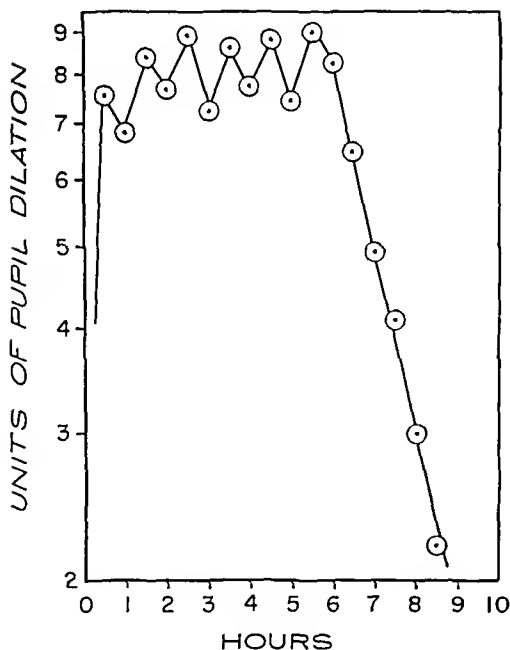


Fig. 3.—Mean pupillary dilation in 12 mice receiving 5 mg./Kg. of SKF 5515-I orally at zero time followed by 1 mg./Kg. at one, two, three, four, and five hours. Each unit of pupil dilation equals approximately 0.2 mm.

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49

NOVEMBER 1960

NUMBER 11

## Solubilization of Riboflavin by Complex Formation with Caffeine, Theophylline, and Dimethyluracil\*

By DAVID E. GUTTMAN and MANOHAR Y. ATHALYE†

The demonstration of complex formation between riboflavin and caffeine by previous workers suggested that xanthines and related compounds might be suitable as solubilizing agents for the vitamin. Caffeine, theophylline, and dimethyluracil were investigated and were found to increase the apparent water solubility of riboflavin. The effect was presumably due to the formation of 1:1 complexes. Apparent dissociation constants were determined to be: caffeine,  $34.5 \times 10^{-3}$  mole/L.; theophylline,  $52.6 \times 10^{-3}$  mole/L.; dimethyluracil,  $182 \times 10^{-3}$  mole/L. The marked difference in complexing tendencies between caffeine and dimethyluracil suggested that the imidazole ring of the xanthine nucleus was strongly involved in the interaction. Quenching of fluorescence experiments were also conducted for comparative purposes.

**R**IBOFLAVIN is a component of many types of multivitamin dietary supplements including vitamin tablets, capsules, oral fluids, and parenterals. Prior to the availability of water-soluble salts of riboflavin-5'-phosphate, the incorporation of the vitamin into the two latter classes of dosage forms was a difficult problem for the pharmaceutical formulator due to its limited solubility in water. In many cases this difficulty was overcome by incorporating an auxiliary agent into the vehicle which had a specific solubility-enhancing effect on riboflavin. The use of these so-called solubilizers has been a common practice and many have been reported in both the scientific and patent literature. Some of the many agents which have been suggested for this purpose are listed in Table I.

Although most of these agents were discovered as a result of empirical investigations, limited evidence indicates that the mechanism of solubilization in a number of cases involved complex formation between riboflavin and the solubilizing agent. Frost (1), for example, inves-

TABLE I—SUGGESTED SOLUBILIZERS FOR RIBOFLAVIN

Agent	Reference
Nicotinamide	(1)
2,4-Dihydroxybenzoic acid and its monoalkyl ether	(2)
Acetamidine HCl, Na <i>p</i> -aminobenzoate, monoethanolamine salicylate, Na <i>p</i> -hydroxybenzoate	(3)
Sodium benzoate, sodium salicylate	(4)
Sodium saccharin	(5)
Gallic acid	(6)
Vanillin	(7)
Veratryl alcohol	(8)
Tyrosine amide	(9)
Salts of hydroxybenzoic acid	(10)
Tryptophan	(11)
3-Pyridylcarbinol	(12)
Na-3-hydroxynaphthoate	(13)
Urea and urethane	(14)
3-Hydroxy-7-sulfo-2-naphthoic acid	(15)
Na desoxycholate, N-methylacetamide	(16)

tigated the effect of nicotinamide, a widely used solubilizer, on the solubility of riboflavin in water. He found that at pH 5, the solubility increased from approximately 0.1 to 2.5 per cent with an increase in nicotinamide concentration from 5 to 50 per cent. At pH values below 5, a

\* Received October 14, 1959, from the College of Pharmacy, The Ohio State University, Columbus 10.

† Present address: Parke, Davis, & Co., Bombay, India.



decrease in this solvent effect was apparent. The results suggested that the solubility-enhancing effect was due in part to salt formation and in part to complex formation.

The interaction of riboflavin with phenol in aqueous solution was studied by Yagi and Matsuoka (17) by both spectral and quenching of fluorescence studies. The near ultraviolet and visible absorption spectrum of riboflavin was found to shift in the direction of longer wavelengths in the presence of phenol. The results were interpreted on the basis of 1:1 complex formation and a dissociation constant of 0.21 mole/L. at 20° was calculated. Complex-formation between the interactants was also suggested by the marked inhibition of the fluorescence of riboflavin in aqueous solution caused by the addition of phenol. Quenching of fluorescence experiments yielded a dissociation constant which was, however, approximately one-ninth that derived from spectral studies. The authors felt that the marked quenching of fluorescence could not be explained solely on the basis of the formation of a nonfluorescent interaction product but must involve other factors. It will be seen that a similar situation was encountered in this investigation.

The effects of various substances on the water-solubility and other properties of riboflavin were investigated by Sakai (18). Compounds such as resorcinol, pyrogallol, hydroquinone, phloroglucinol, *p*-aminobenzoic acid, anthranilic acid, phthalic acid, benzoic acid, and others were found to exhibit solubilizing action. Related aliphatic compounds did not. Hydrogen-bond formation was postulated between the vitamin and some of the solubilizers. Harbury and Foley (19), however, studied the interactions of various isoalloxazine derivatives and conjugated molecules by spectral measurements and determined that in several systems where no possibility of hydrogen-bond formation existed an interaction did, in fact, occur. They suggested that hydrogen-bonding is not a primary feature of such interactions but that molecular charge transfer and other mechanisms may be more important. The results of the present investigation support this view.

The objective of this investigation was to study caffeine and some related compounds as potential solubilizers of riboflavin. It was expected, on the basis of structural similarities between riboflavin and other compounds which have been shown to complex with caffeine, that interactions and subsequent solubilization would occur. A search of the literature revealed that complex

formation between caffeine and riboflavin and between caffeine and 3-methylriboflavin was indeed known. Quenching of fluorescence studies and spectral studies were utilized by Weber (20) and Yagi and Matsuoka (17), respectively, to determine the dissociation constant of a 1:1 complex which formed between caffeine and riboflavin. The value of 0.019 mole/L. at 20° determined by the latter workers did not agree with the value of 0.011 mole/L. obtained by Weber at approximately the same temperature. Harbury and Foley (19) studied the caffeine-3-methylriboflavin system and determined by spectral studies a dissociation constant of 0.021 mole/L. at 22.5°. These constants must be considered as approximate since the nonideal nature of caffeine in aqueous solution was not recognized by the investigators.

Of pharmaceutical interest is the application of these interactions to increase the apparent solubility of riboflavin in a dosage form. Although previous workers were not motivated by this objective, their results recommend this extension. For example, the dissociation constant for the riboflavin-caffeine complex as given by Yagi and Matsuoka suggests that the solubility of riboflavin in a 2 per cent solution of caffeine would be approximately six times that in water alone. The present investigation was designed to test the anticipated riboflavin-solubilizing ability of caffeine, theophylline, and dimethyluracil and to gain additional information concerning the nature of the interactions.

## RESULTS

**Solubility Studies.**—Solubilization through complex formation is attributed to the formation of a new species in the solution phase which has a solubility, independent of that of the parent compound. In such a system, two simultaneous equilibria are operative. One involves the reversible transfer of solute from the solid phase to the solution phase while the other is the reversible association of interactants to form the complex. From this consideration it can be shown that in a system where a complex of 1:1 stoichiometry is formed, solubility relationships can be described by

$$S/S_0 = 1 + (C/K) \quad (\text{Eq. 1})$$

where  $S_0$ =solubility of solute in the absence of complexing agent,  $S$ =apparent solubility of solute in the presence of the complexing agent,  $C$ =molar concentration of complexing agent, and  $K$ =dissociation constant of the complex.

Equation 1 assumes that activities of the species can be represented by concentration terms and that the activity of the solvent is not markedly affected by the presence of complex and/or complexing agent. If the stoichiometric concentration of complexing agent is large compared to the concentra-

tion of complex, then, Eq 1 can be approximated by

$$S/S_0 = 1 + (C_i/K) \quad (\text{Eq 2})$$

where  $C_i$  = stoichiometric concentration of complexing agent

Equation 2 thus provides a graphical method for the determination of the dissociation constant

Results of the solubility studies are shown in Fig 1 where the function  $S/S_0$  is plotted as a function of the concentration of complexing agent. Definite interactions are indicated by the results. It can be seen, for example, that the solubility of riboflavin in a 0.0927 *M* solution of caffeine in water exceeded that in water alone by a factor of three. No difference in solubilizing action was found when 0.087 *M* acetic acid was used in place of distilled water. This suggests that the interaction was independent of pH, at least in the acid to neutral range.

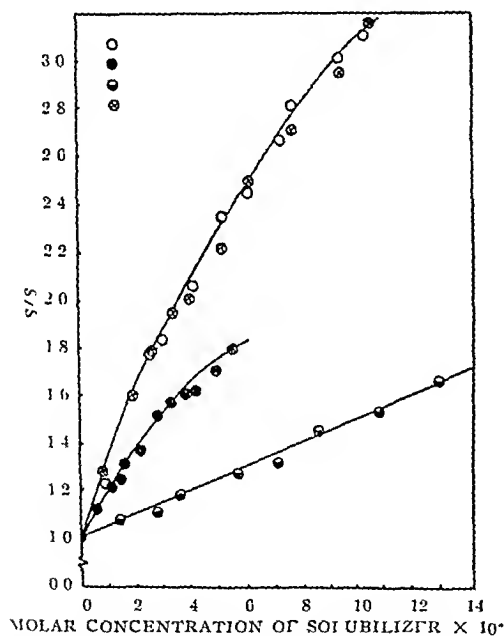


Fig 1—The solubility of riboflavin in solutions of caffeine, theophylline, and dimethyluracil at 30°. O, Caffeine, ●, theophylline, ⊙, dimethyluracil, ⊗, Caffeine at pH 4.2

Marked deviations from the expected linearity of this plot are apparent in the case of caffeine and theophylline. The deviations are not unexpected in view of the reported nonideal behavior of caffeine and theophylline in aqueous solution (21). The deviations are such that they can be explained on this basis. In dilute solutions where the xanthines existed in essentially monomeric form, deviations from linearity were small. At higher concentrations, where dimeric and tetrameric species predominate, the deviations were correspondingly marked. It was interesting to observe that linearity throughout a wide range of concentration held for dimethyluracil. This behavior strongly suggests that in contrast to caffeine and theophylline, dimethyluracil does not

undergo marked self complexing in aqueous solution. Because of this, dimethyluracil might be a desirable agent for basic studies of caffeine type complex formation.

Dissociation constants were calculated from the slopes of the curves of Fig 1. The initial straight line portions of the curves representing caffeine and theophylline were used for this purpose. The values obtained must be assumed to represent apparent dissociation constants which are valid only for comparative purposes. The constants are tabulated in Table II. It is seen that the tendency for complex formation is greatest with caffeine and significantly smaller with dimethyluracil. The constant calculated for the caffeine-riboflavin complex is not in agreement with values determined by previous workers, especially that of Weber. The degree of complex formation indicated by Weber's study was much greater than that determined in the present investigation. This discrepancy motivated a further study, essentially an extension of Weber's work, in attempt to explain the lack of agreement between experimental methods.

TABLE II—DISSOCIATION CONSTANTS OF SOME COMPLEXES OF RIBOFLAVIN AT 30° ± 0.5°

Complexing Agent	Dissociation Constant Mole/l
Caffeine	$34.5 \times 10^{-3}$
Theophylline	$52.6 \times 10^{-3}$
Dimethyluracil	$182 \times 10^{-3}$

**Quenching of Fluorescence Studies**—A common observation is quenching of fluorescence, a reduction in or an elimination of the fluorescence of compounds, caused by changes in temperature, solvent, physical state, or by the presence of quenching agents. The mechanism of action of the latter can be quite complex. The quenching effect can be due to collisions between a quencher and the fluorescent species, or to a nonspecific transient interaction between them, or a combination of the two effects. Alternatively, apparent quenching can result from a strong interaction which results in the formation of a nonfluorescent complex.

The marked quenching effect exhibited by caffeine on the fluorescence of riboflavin in aqueous solutions is illustrated in Fig 2. Here the intensity of fluorescence of a solution containing a riboflavin concentration of 1 meg/ml is plotted versus the concentration of caffeine in the solution. It can be seen that at a caffeine concentration of 0.1 mole/l, for example, an approximate 90% decrease in fluorescence occurred. Similar results were obtained at riboflavin concentrations of 0.50 and 0.25 meg/ml.

If the observed quenching effect was due solely to the formation of a nonfluorescent complex of 1:1 stoichiometry, then, according to the treatment of Weber, it can be shown that

$$I_0/I = 1 + (\text{Caffeine}/K) \quad (\text{Eq 3})$$

where  $I_0$  = intensity of fluorescence in the absence of caffeine,  $I$  = intensity of fluorescence in the presence of caffeine, caffeine = molar concentration of caffeine, and  $K$  = dissociation constant of the complex.

If the concentration of caffeine is large compared

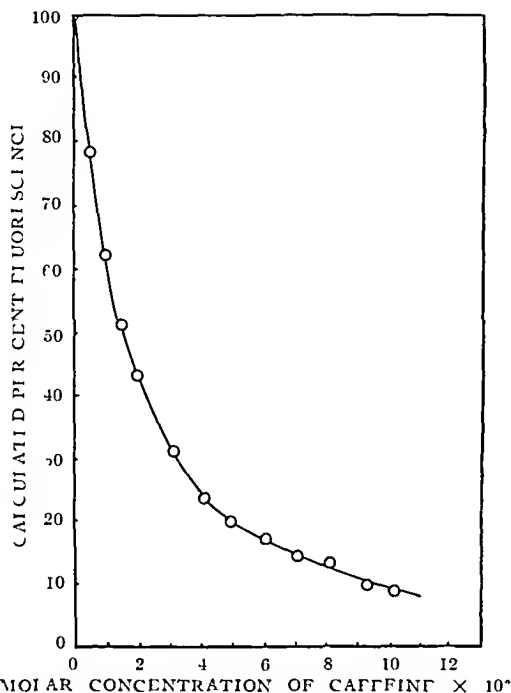


Fig 2—The quenching of riboflavin fluorescence by caffeine. Riboflavin concentration = 1 mcg/ml

to that of the complex, Eq 3 can be approximated by

$$I_0/I = 1 + (\text{Caffeine}_i/K) \quad (\text{Eq 4})$$

where  $\text{Caffeine}_i$  = stoichiometric concentration of caffeine

A plot of  $I_0/I$  versus (caffeine)<sub>i</sub> provides the means to test the assumptions and to determine the dissociation constant graphically. The results with caffeine at three different concentrations of riboflavin are plotted in this manner in Fig 3. The lack of linearity of the experimental curves and the different curves obtained at the different concentrations of riboflavin indicate that quenching is not entirely due to the formation of a nonfluorescent complex. The upward curvature of the lines strongly suggests that other quenching mechanisms were also operative. The dissociation constant of  $14.6 \times 10^{-3}$  mole/L calculated from the initial linear portion of the curves approximates that determined by Weber. This agreement indicates the reproducibility of the method but does not substantiate the validity of the basic premises. The quenching effects of theophylline and dimethyluracil were also investigated and the results are summarized in Fig 4 where a caffeine curve was also plotted for comparative purposes. It will be noticed that this method failed to distinguish between the complexing tendencies of caffeine and theophylline. A definite difference was apparent from solubility experiments. Although the curve for dimethyluracil is linear, the dissociation constant calculated from this plot is only one fifth that obtained from solubility experiments. In all cases the fluorescence studies indicated a much greater degree of complex formation than correspond-

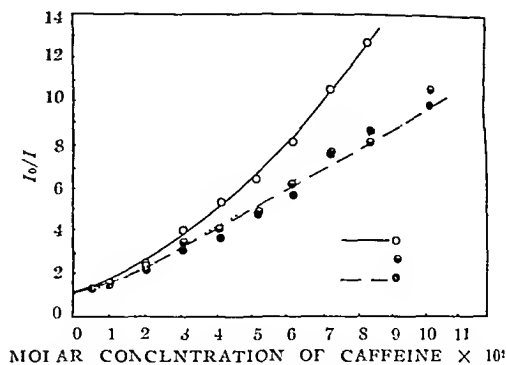


Fig 3—The quenching of riboflavin fluorescence by caffeine. Riboflavin concentration: O, 0.25 mcg/ml, O, 0.50 mcg/ml, ●, 1.00 mcg/ml

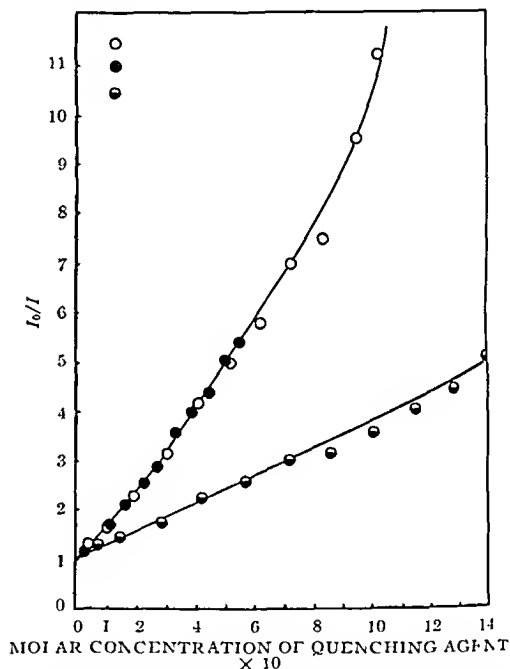
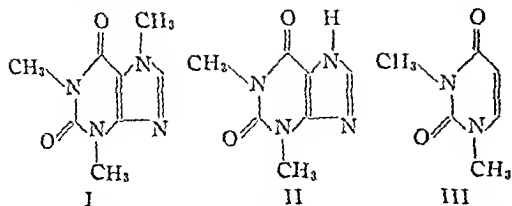


Fig 4—The quenching of riboflavin fluorescence by caffeine, theophylline, and dimethyluracil. Riboflavin concentration = 1 mcg/ml. O, Caffeine, ●, theophylline, O, dimethyluracil

ing solubility studies. Complexing of riboflavin by these agents undoubtedly contributes in part to the overall degree of quenching, however, additional mechanisms must also be operative. Similar observations were made by Yagi and Matsuoka (17) on the phenol-riboflavin system.

## DISCUSSION

Some insight into the nature of the interaction between riboflavin and the three compounds studied can be obtained by comparing complexing tendencies. The stabilities of the complexes formed with caffeine (I) and theophylline (II) were found to be many times greater than that of dimethyluracil (III)



The imidazole ring of the xanthine nucleus must then be strongly involved in the interaction. This supports the suggestion of Harbury and Foley (19) that hydrogen bonding may not be the main interaction component of isalloxazine-xanthine complexes. The three compounds studied in this investigation all contain the 1,3-dimethylpyrimidin-2-one nucleus, a potential center for hydrogen-bond formation. Nevertheless, the stability of the caffeine complex is approximately five times that of the dimethyluracil product. It is possible, however, that hydrogen bonding provides a primary linkage between the interactants and is reinforced by secondary van der Waal interactions which involve other parts of the molecule. Interpretations are equivocal however, in view of the limited data available.

It is interesting to compare caffeine with other compounds reported in the literature as solubilizers of riboflavin. The solubility of riboflavin in 0.1 *M* solutions of nicotinamide (1), vanillin (7), gallic acid (6), and acetaminide HCl (3) were interpolated from the data presented in the references to be  $0.66 \times 10^{-3}$ ,  $0.60 \times 10^{-3}$ ,  $0.69 \times 10^{-3}$ , and  $0.25 \times 10^{-3}$  mole/L., respectively. In comparison, the solubility in a solution of caffeine of corresponding concentration was  $1.27 \times 10^{-3}$  mole/L. It seems quite probable that structural modifications of the xanthine nucleus might yield compounds capable of more pronounced solubilizing action by virtue of stronger complexing tendencies.

## EXPERIMENTAL

**Materials.**—Riboflavin was obtained from Eastman. Caffeine and theophylline were U S P grade. Dimethyluracil was synthesized according to the procedure of Davidson and Baudisch (22).

**Solubility Studies.**—Ten-milligram quantities of riboflavin were weighed into a series of 25-ml glass-stoppered Erlenmeyer flasks. Distilled water and/or a solution of the agent under investigation was added by buret to each flask to make a total volume of 20 ml. The flasks were stoppered, placed in a constant temperature bath at  $30 \pm 0.5^\circ$ , and agitated for sixteen hours. Agitation was stopped and the undissolved powder was permitted to settle. An aliquot of each solution was removed and rapidly filtered with the aid of a Swinney filter adaptor attached to a syringe. One milliliter of the filtrate was

diluted with distilled water to 500 ml in a volumetric flask. Approximately 50 ml. of the solution was extracted three times, each with 20 ml. of chloroform. Preliminary experiments showed that this method effected the desired extraction of caffeine and that riboflavin was not extracted from its aqueous solution. The aqueous solution of riboflavin was then gently warmed on a water bath to remove any trace of chloroform. This precaution was necessary since chloroform was found to exert a slight quenching effect. The concentration of riboflavin was then determined fluorimetrically with a Lumetron fluorimeter. The instrument was adjusted to 100% intensity with a 1.5 meg./ml solution of riboflavin. A standard curve was obtained by using known concentrations of the vitamin. This curve was used to determine the riboflavin content of all solutions.

**Quenching of Fluorescence Studies.**—A solution containing 50 meg./ml of riboflavin was prepared according to the directions of the "United States Pharmacopeia XV" (23). An aliquot of this was transferred to each of a series of 100-ml volumetric flasks. Distilled water and/or a solution of the agent under investigation was added by buret to each flask to make a total volume of 100 ml. The fluorescent intensity of each solution was then determined with a Lumetron fluorimeter.

## REFERENCES

- (1) Prost, D. V., *J. Am. Chem. Soc.*, **69**, 1064 (1947).
- (2) Hoffmann-La Roche, Brit. pat. 555,346 (1943), through *Chem. Abstr.*, **39**, 710 (1945).
- (3) Jurist, A. E., U. S. pat. 2,358,331 (1944), through *Chem. Abstr.*, **39**, 1514 (1945).
- (4) Miller, H. C., U. S. pat. 2,395,378 (1946), through *Chem. Abstr.*, **40**, 2593 (1953).
- (5) Terao, K., Jap. pat. 173,506 (1916), through *Chem. Abstr.*, **46**, 2242 (1952).
- (6) Bird, J. C., and Kuna, A., U. S. pat. 2,407,624, through *Chem. Abstr.*, **41**, 569 (1947).
- (7) Charney, J., U. S. pat. 2,449,640 (1948), through *Chem. Abstr.*, **42**, 9094 (1948).
- (8) Knaufet, A. E., and Kirchmeyer, F. J., U. S. pat. 2,440,050 (1949), through *Chem. Abstr.*, **42**, 4722 (1948).
- (9) Charney, J., U. S. pat. 2,445,208 (1948), through *Chem. Abstr.*, **42**, 6496 (1948).
- (10) Ferrosan, A., Dan. pat. 64,000 (1945), through *Chem. Abstr.*, **40**, 4184 (1946).
- (11) Harter, R. A., and Chen, J. L., *THIS JOURNAL*, **38**, 568 (1949).
- (12) Schlapter, R. U. S. pat. 2,458,430 (1949), through *Chem. Abstr.*, **43**, 1914 (1949).
- (13) Suter, C. M., U. S. pat. 2,601,569, through *Chem. Abstr.*, **46**, 9808 (1952).
- (14) Schou, S. A., and Fretheim, B., *Dansk Tidsskr. Farm.*, **14**, 97 (1940).
- (15) Auerbach, M. A., U. S. pat. 2,837,461 (1958), through *Chem. Abstr.*, **52**, 17631 (1958).
- (16) Kuhn, K., *Klin. Wochschr.*, **17**, 222 (1938).
- (17) Yagi, K., and Matsuoka, Y., *Biochem. Z.*, **328**, 138 (1956).
- (18) Sakai, K., *Nagoya J. Med. Sci.*, **18**, 237 (1956).
- (19) Harbury, H., and Foley, K. A., *Proc. Natl. Acad. Sci. U. S. A.*, **44**, 662 (1958).
- (20) Weber, G., *Biochem. J.*, **47**, 114 (1950).
- (21) Guttman, D., and Higuchi, T., *THIS JOURNAL*, **46**, 4 (1957).
- (22) Davidson, D., and Baudisch, O., *J. Am. Chem. Soc.*, **48**, 2382 (1926).
- (23) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 612.

# Antibacterial Activity of Essential Oil Vapors<sup>\*</sup>

By JASPER C. MARUZZELLA and NICHOLAS A. SICURELLA

Vapors of 133 essential oils were screened *in vitro* for antibacterial activity against six test organisms. One hundred and five vapors were found to possess antibacterial activity on at least one of the test organisms. Vapors of thyme (white), cassia, thyme (red), savory, cinnamon, oregano, and cherry laurel produced prominent zones of inhibition with all test organisms. Gram-positive bacteria were more susceptible to the vapors than Gram-negative bacteria.

**S**TUDIES on the action of essential oil vapors on anthrax spores were reported in 1887 by Chamberlain (1). Subsequent investigations by Greig Smith (2), Macht and Kunkel (3), Morel and Rochaix (4, 5), Schobl and Kusama (6), Coulthard (7), Remlinger and Bailly (8), and recently by Ru (9) and Grubb (10) have clearly demonstrated that essential oil vapors possess antibacterial properties. Yet the proper evaluation of these volatile plant products on bacteria cannot be fully realized because of the diverse methods used by various investigators and the relatively small number of essential oil vapors tested. This study was undertaken in an attempt to evaluate a large number of essential oil vapors against a variety of Gram positive and Gram negative bacteria.

## MATERIALS AND METHODS

Vapors of 133 essential oils were screened for antibacterial activity against the following test organisms: *Escherichia coli* (B strain), *Staphylococcus aureus* (ATCC 10390), *Bacillus subtilis* var. *aterrimus* (ATCC 6461), *Streptococcus fecalis*, *Salmonella typhosa* (ATCC 10794), and *Mycobacterium avium* (ATCC 4676). Twenty-four hour cultures of these organisms were grown in nutrient agar and broth (Difco) at 37° except for *M. avium* which was grown for seventy-two hours in 5% glycerol broth and agar.

The detection of essential oil vapors for antibacterial activity was conducted in the following manner. Approximately 18 cc of melted agar were poured into Petri dishes and allowed to solidify. To the surface of the agar was added 0.2 cc of bacterial broth culture which was spread with a glass spreader with the aid of a turntable. Filter paper disks (6.35 mm diameter) were completely saturated with the oil to be tested and placed in the center of the inner surface of the Petri dish cover. Thus, when the dishes were inverted and incubated, the saturated disks were at a distance of about 8 mm

from the surface growth of the organism. All dishes were incubated at 37° for twenty-four hours except those with *M. avium*, which were incubated for seventy-two hours. The presence of a clear zone on the surface of the agar above the disk was indicative of antibacterial activity, and degree of activity was determined by the size of the zone of inhibition. The diameter of the zones of inhibition was measured to the nearest millimeter by means of a metric ruler with the aid of an illuminated Quebec colony counter. All plates were conducted in quadruplicate with one disk per dish. Each measurement therefore represents a mean value of four recordings. While it is recognized that day to day fluctuations in the resistance of the test organisms may have occurred, they are not considered to affect the overall screening of the vapors against the bacteria appreciably.

## RESULTS AND DISCUSSION

Vapors of 133 essential oils were used in conducting 784 tests on the six test organisms. In this series of tests, the vapor showed antibacterial activity in 179 (23%) cases. Table I lists the essential oil vapors together with their zones of inhibition. Of the 133 oil vapors tested, 71% were found to be active against *M. avium*, 19% against *B. subtilis* var. *aterrimus*, 12% against *S. fecalis* and 6% against *E. coli*. Of the one hundred and nineteen oil vapors screened against *S. aureus*, 14% showed activity. Thus, *M. avium* was the most susceptible organism while *E. coli* was the most resistant. Moreover, the Gram positive bacteria were more vulnerable to essential oil vapors than the Gram negative.

Further inspection of the data reveals that the vapors of the following oils produced the largest zones of inhibition on all test organisms: thyme

TABLE I—INHIBITORY ACTIVITY OF ESSENTIAL OIL VAPORS

Oils	Diameter of Zones of Inhibition mm				
	<i>S. aureus</i>	<i>B. subtilis</i> var. <i>aterrimus</i>	<i>S. fecalis</i>	<i>S. typhosa</i>	<i>M. avium</i>
<i>Abies alba</i> (from needles)	0	0	0	0	90
<i>Abies Sibirica</i>	0	0	0	0	67
Anise	0	0	0	0	46
Angelica root	0	0	0	0	71
Angelica seed	0	0	0	0	29
Anise, U. S. P.	0	0	0	0	54
Basil, sweet	0	0	0	0	32
Bay N. F.	0	0	0	0	44
Bergamot N. F.	0	0	0	0	72
Birch tar, rectified	0	0	0	0	36
Bois de rose	0	0	0	19	90
Citric, rectified U. S. P.	0	0	0	0	64
Cajuput	0	0	0	0	62

\* Received February 2, 1960 from the Department of Biology, Long Island University, Brooklyn, N. Y.

This paper is based upon a thesis submitted to the Graduate School of Long Island University by Nicholas A. Sicurella in partial fulfillment of the requirements for the degree of Master of Science.

TABLE I.—(Continued)

Oils <sup>a</sup>	Diameter of Zones of Inhibition, mm. <sup>b</sup>					Oils <sup>a</sup>	Diameter of Zones of Inhibition, mm. <sup>b</sup>				
	<i>S. aureus</i>	<i>B. subtilis</i> var. <i>alerrimus</i>	<i>S. fecalis</i>	<i>S. typhosa</i>	<i>M. avium</i>		<i>S. aureus</i>	<i>B. subtilis</i> var. <i>alerrimus</i>	<i>S. fecalis</i>	<i>S. typhosa</i>	<i>M. avium</i>
Cananga, rectified	0	0	0	0	60	Palmarosa	0	0	0	0	46
Caraway	.. <sup>d</sup>	0	0	0	60	Patchouly, Singapore	0	28	0	0	60
Cardanum	0	0	0	0	53	Pennyroyal, imported	..	0	0	0	65
Cascarilla	0	0	0	0	32	Pepper, black	0	0	0	0	46
Cassia U. S. P.	25	22	44	19	60	Peppermint, natural	8	17	0	0	90
Cedar leaf	..	23	0	0	70	Peppermint, rectified	10	0	0	0	63
Cedar wood	0	29	0	0	68	U. S. P.					
Celery seed	0	0	0	0	20	Petitgrain, Paraguay	0	0	0	0	65
Chenopodium N. F.	0	0	0	0	69	Pimenta (from berries)	0	0	0	22	43
Cherry laurel	..	0	90	90	90	Pimenta leaf	0	0	0	29	38
Cinnamon, Ceylon	28	20	31	24	54	Rhodium	0	0	0	7	71
Citronella, Formosan	0	54	0	0	90	Rose, kasanlik, U. S. P.	0	0	0	0	43
Clove U. S. P.	0	0	0	22	43	Rosemary, N. F.	..	0	0	0	25
Clove leaf, rectified	0	0	0	0	31	Rosemary, acetylated	..	0	0	0	63
Cognac green	0	0	0	0	64	Rue	0	0	0	0	90
Copaiba	0	0	0	0	30	Rusei, rectified	0	0	0	0	56
Coriander U. S. P.	0	9	0	21	70	Sage, Dalmatian	..	16	0	0	69
Cubeb	0	15	0	0	60	Sage, Clary	0	0	0	0	62
Cumin	..	6	0	0	70	Sandalwood	0	0	0	0	25
Curacao peel	0	0	0	0	16	Sassafras N. F.	0	0	0	0	37
Cypress	0	0	0	0	50	Savin	0	0	0	0	72
Dill seed	0	0	0	0	90	Savory select	34	24	17	17	68
Dill weed	..	0	0	0	67	Snakeroot, Canada	0	0	0	0	27
Erigeron	0	0	0	0	22	Spearmint, N. F.	..	0	0	0	54
Estragon	0	0	0	0	25	Spike lavender	15	0	0	0	74
Eucalyptus, rectified N. F.	0	0	17	0	62	Spike lavender, acetylated	0	0	0	0	90
Fennel U. S. P.	0	0	0	0	68	Spruce	0	18	0	0	90
Galbanum	..	0	0	0	33	Sweet birch, northern	0	0	0	0	90
Garlic, imported	.. <sup>e</sup>	24	0	0	90	U. S. P.					
Geranium, Algerian	0	0	0	0	61	Tansy	..	0	0	0	72
Ginger	0	0	0	0	22	Tar, rectified, N. F.	0	0	0	0	68
Gingergrass, native	0	0	0	0	57	Thyme, red N. F.	25	28	25	25	57
Hemlock	0	0	0	0	64	Thyme, white N. F.	26	24	20	27	51
Labdanum	..	0	0	0	22	Ti-Tree, Australian	0	0	0	0	69
Laurel leaf	0	0	0	0	29	Turpentine, rectified N. F.	10	0	21	18	60
Lavandin	0	0	0	0	90	Valerian, Indian	10	10	7	0	34
Lavender U. S. P.	0	0	0	0	82	Verbena	10	17	0	0	90
Lemongrass, rectified	33	42	29	0	90	Vetiver, Haiti	0	0	0	0	14
Linaloe wood	0	0	0	12	73	Wintergreen, northern	0	0	0	0	90
Mace	0	0	0	0	44	U. S. P.					
Majoram, sweet	0	11	0	0	68	Wormwood	..	0	0	0	66
Melissa balm (so-called)	0	0	0	0	67	Ylang Ylang	0	0	0	0	29
Mountain laurel	0	0	0	0	36						
Myrtle (so-called)	0	0	0	0	62						
Neroli, bigarade petale, N. F.	0	0	0	0	66						
Nutmeg, East Indian U. S. P.	0	0	0	0	62						
Nutmeg West Indian U. S. P.	0	0	0	0	24						
Ocotea Cymbarum	0	0	0	0	27						
Olibanum	..	0	0	0	33						
Onion	..	26	0	0	90						
Orange, bitter	0	0	0	0	21						
Origanum	18	20	17	19	39						
Origanum, rectified water white	18	22	20	22	49						

<sup>a</sup> The essential oils were generously supplied by Magnus, Mabey, and Reynard, Inc., New York, and Fritzsche Brothers, Inc., New York.

<sup>b</sup> The following essential oil vapors were active against *E. coli*: cassia U. S. P. (20 mm.), cherry laurel (90 mm.), cinnamon (Ceylon) (19 mm.), eucalyptus, rectified N. F. (15 mm.), origanum (21 mm.), savory, select (20 mm.), thyme red N. F. (28 mm.), and thyme white N. F. (29 mm.).

<sup>c</sup> Zone of inhibition absent.

<sup>d</sup> Not tested.

<sup>e</sup> Reduced growth as compared to control with no definite zone of inhibition.

(white), cassia, thyme (red), savory, cinnamon (Ceylon), organum, and cherry laurel

The 28 oil vapors with no activity were *Abies alba* (from cones), amber, rectified, balsam Peru, calamus, camphor, sassafras, camphor, white, chamomile, German, dwarf pine needle, grapefruit, Florida expressed, guaiac wood, hops, juniper, twice rectified, lemon, California coldpressed U S P, lemon, Italian handpressed U S P, lime, distilled, lime, expressed, lovage, mandarin, Italian, myrrh, maouli, opopanax, orange, California sweet, coldpressed U S P, orris root, Florentine, parsely seed, persic U S P, *Pinus sylvestris*, styra, and tangerine.

Why *M. avium* should be so susceptible to essential oil vapors is not known. One possible explanation might be the fact that this organism was exposed to the vapors for a longer period of time (seventy-two hour incubation period) and hence

greater susceptibility due to greater absorption of the vapors on to the agar. One might speculate as to the possible mode of action involving a penetration of the essential oil vapor into the cell barrier disrupting the chemical organization (lipids) of the cell

## REFERENCES

- (1) Chamberlain, M, *Ann Inst Pasteur*, 1, 153(1887)
- (2) Greig Smith, R, *Proc Linn Soc N S Wales*, 44, 72(1919)
- (3) Macht, D I, and Kunkel, W M, *Proc Soc Exptl Biol Med*, 18, 68(1920)
- (4) Morel, A, and Rochaix, A, *Compt rend soc biol*, 85, 861(1921)
- (5) Morel, A, and Rochaix, A, *Parfumerie mod*, 18, 261(1925)
- (6) Schöbl, O, and Kusama, H, *Philippine J Sci*, 24, 443(1924)
- (7) Coulthard, C H, *Brit J Exptl Pathol*, 12, 331(1931)
- (8) Remlinger, P, and Bailly, J, *Ann Inst Pasteur*, 68, 428(1942)
- (9) Ryu, E, *Kyoto Arch Exptl Med*, 29, 37(1956)
- (10) Grubb, T C, *This Journal*, 68, 272(1959)

# Growth and Differentiation of *Atropa belladonna* L. as Affected by Different Sources of Nitrogen<sup>\*</sup>

By L. J. SCHERMEISTER†, F. A. CRANE, and R. F. VOIGT

Growth and morphology were compared on plants furnished six levels of nitrate and three of ammonia in water culture. The production of dry weight with ammonia was significantly greater than with nitrate. Ratios of shoot/root and leaf/stem indicated that use of the nitrogen was very different at low vs. high levels, particularly with ammonia nitrogen.

MANY LITERATURE reports relating the use of fertilizer combinations to alkaloid production in the solanaceous drug plants have led to a vague understanding of what the plant needs and how it is used. In this study several different nitrogenous sources were furnished belladonna plants grown in nutrient solutions and the patterns of growth and maturation were observed and described.

Growth studies reported on the belladonna plant indicate that a nitrogen source furnished early enough in the vegetative growth period significantly increases plant production and stimulates alkaloid synthesis (1-7). They did not make clear the advantages of one form of nitrogen over another nor did they deal with the problem of availability of the nutrients to the plant from the various types of manures or soils in which the plants were grown. The reference

of James (4) to the improper balance of fertilizer elements further points up the need for growing the plants in a controlled medium in which purified chemicals are the only nutrients available. Since the belladonna plant is capable of synthesizing its nitrogenous constituents from inorganic sources, plants were grown in nutrient solutions containing several concentrations of nitrate and ammonium salts. Plants were grown through a standard growth period to a definite flowering stage, were harvested, and representative samples were prepared for the various determinations reported later. During the growth periods measurements were made that characterize the features of this plant as they were affected by nutrition. Ratios of various plant parts to each other indicate the effect of nutritional change on morphology.

## EXPERIMENTAL PROCEDURE

Plants used in all experiments were germinated from good quality acid-scarified seed in a sand-vermiculite mixture maintained at 70° F. Germination occurred within two to three weeks

<sup>\*</sup> Received September 25, 1959, from the University of Illinois, College of Pharmacy, Chicago 12.

This paper is based on a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree Doctor of Philosophy.

† Present address: School of Pharmacy, North Dakota Agricultural College, Fargo.

Following germination, the young seedlings were transferred to a full nutrient solution (8) where they were prepared for the experimental treatments.

Plants were grown in 3-gallon glazed crocks, supported on specially designed masonite covers. They were lightly fastened by plant ties to the wire frame as their size required.

Nutrient solutions were prepared from reagent grade chemicals and distilled water on a mg. equiv. per L. basis. The composition of nutrient solutions is shown in Table I. The levels of nitrogen were varied both above and below the level of the well-known Hoagland and Arnon solution (15 mg. equiv./L.) which was considered a standard or control concentration. A preliminary experiment had shown that belladonna plants could not tolerate ammonia as a sole nitrogen source in culture solution. Therefore, ammonia at three levels was supplemented by nitrate in a 1-4 ratio as shown in Table I. Distilled water was added periodically to maintain volumes and the pH of solutions was adjusted to 5.8 weekly with dilute hydrochloric acid.

TABLE I.—COMPOSITION OF NUTRIENT SOLUTIONS USED FOR GROWING *Atropa belladonna* L.<sup>a</sup>

All solutions contained the following ions mg. equiv./L.	
Calcium..... 10	Phosphorus..... 1
Potassium..... 4	Sulfur..... 4
Magnesium..... 4	Trace elements (H and A mixture).....
Iron (as citrate)..... 1 per week	..... 1 per week
Experimental solutions contained in addition to those above	
Nitrogen as Nitrate..... 5, 10, 15, 20, 25, 30	
Nitrogen as ammonium and as nitrate..... 1 and 4, 4 and 16, 6 and 24	

<sup>a</sup> These nutrient solutions were prepared from Gm. equiv. stock solutions of the following salts, by adding 1.0 ml. of the stock solution for each mg. equiv. desired in the final solution to water, and made up to the desired volume. Calcium nitrate (Sorensen's), acid phosphate chloride, ferric citrate (0.5%), without nitrate the corresponding chloride was used, since chloride has not been proved essential for plant growth.

Measurements of shoot length, number of initiated and elongated leaves, flowering shoots, and lateral vegetative shoots were made weekly during the growth period. Plants were harvested at a selected stage, i. e., when 50% of the flower buds of the plants at a particular nitrogen level had opened. Fresh weights were taken at harvest on all plants. Representative samples were taken for the various nitrogen determinations and all other plants were dried (air dried for several days, then two hours at 100°) for dry weights. Samples of the nutrient solutions were analyzed after harvesting to determine actual uptake of nitrogen by the plants.

## EXPERIMENTAL RESULTS

There was a regular progression of growth increases with increased nitrogen supply even to the maximum used (Fig. 1 and Table II). This would indicate that the plants might properly use nitrate or ammonia in the greater amount, though the dry weights of leaves and shoots (Table III) indicate that at the upper level of nitrogen supply less photosynthetic products are accumulated, rendering nitrogen levels higher than that which was used undesirable.

The regular progression of shoot elongation with time was of the same order of magnitude as the fresh weights of the plants at the time of harvest. In each case the  $\text{NH}_3\text{-NO}_3$  plants were somewhat larger than those of the corresponding level of  $\text{NO}_3$ .

The number of leaves produced appeared to be independent of changes in the nitrogen supply, since growth involved production of leaves by apical meristems and the pattern of elongation which followed the leaf initiation was the phase that reflected the nutritional differences. However, branching and flowering (Table IV) were markedly influenced by the nitrogen source. At high nitrogen levels, particularly  $\text{NH}_3\text{-NO}_3$ , the number of branches produced and the number of flowers per branch were greatly increased (50 flowers vs. 20-30



Fig. 1.—Plants of *Atropa belladonna* L. grown in nutrient solutions that contained (upper row) 5, 10, 15, 20, 25, 30 mg. equiv./L. of nitrate nitrogen, and (lower row) 5, 20, 30 mg. equiv./L. of ammonia-nitrate nitrogen.



TABLE II—FRESH WEIGHT OF SEPARATED PARTS OF BELLADONNA PLANTS<sup>a</sup>

Solution Composition mg equiv. of N/L	Leaf	Stem	Root	Flowers	Total Plant
Nitrate					
5	20.5	33.6	26.8	4.6	85.5
S D	5.2	12.8	8.6	3.3	29.7
10	32.1	43.0	27.2	5.9	108.2
S D	14.3	19.1	9.0	3.4	39.2
15	39.6	46.1	33.1	6.7	125.5
S D	13.1	18.6	2.5	4.4	39.6
20	43.1	46.2	33.5	7.6	130.4
S D	10.9	15.7	9.9	5.4	41.4
25	48.0	46.6	33.5	6.6	134.7
S D	11.0	18.0	14.1	5.1	46.1
30	51.9	48.0	36.2	5.7	141.8
S D	15.0	18.0	18.6	4.2	53.1
Ammonia-Nitrate					
5	19.0	31.1	30.1	4.9	85.1
S D	5.3	12.1	12.0	2.4	30.8
20	51.6	55.8	36.7	9.1	153.2
S D	13.8	17.7	10.5	5.2	43.9
30	57.8	61.2	40.1	12.8	171.9
S D	10.4	13.6	12.2	5.5	35.8

<sup>a</sup> Weights in Gm. are means of 12 plantsTABLE III—DRY WEIGHT OF SEPARATED PARTS OF BELLADONNA PLANTS<sup>a</sup>

Solution Composition, mg equiv. of N/L	Leaf	Stem	Root	Flowers	Total Plant
Nitrate					
5	3.44	5.84	3.10	0.88	13.26
S D	0.53	0.50	0.23	0.11	0.45
10	4.20	8.43	3.15	1.30	17.08
S D	1.39	1.02	0.68	0.30	1.50
15	6.33	8.43	3.57	1.40	19.73
S D	0.52	1.03	0.36	0.24	1.45
20	7.32	9.23	3.92	1.58	22.05
S D	0.81	1.01	0.58	0.42	1.09
25	6.02	8.11	3.37	1.32	18.82
S D	0.44	2.72	0.63	0.79	4.05
30	6.46	7.96	3.65	1.23	19.30
S. D.	0.96	0.47	0.42	0.22	1.53
Ammonia-Nitrate					
5	3.04	5.12	3.69	1.00	12.85
S D	0.76	0.63	0.39	0.20	1.74
20	7.44	10.46	3.98	1.82	23.70
S D	0.49	0.56	0.31	0.38	0.72
30	7.73	11.40	4.27	2.40	25.80
S D.	0.61	0.36	0.54	0.64	1.34

<sup>a</sup> Weights in Gm. are means of 12 plants

at lower nitrogen levels), reflecting the essential need for the constituents of protein in the synthesis of new tissues. Regular increase of fresh weight to the highest level of both nitrate and nitrate-ammonia treatments in all plant parts except flowers, further indicates the high requirement of nitrogen for satisfactory plant synthesis. The reduced weight of flowers at high nitrate indicates that high nitrogen favors vegetative over reproductive growth.

Dry weights varied between 15 and 20% of fresh weights (Tables II, III). At low  $\text{NO}_3$  and  $\text{NH}_4\text{-NO}_3$  levels nutritional effects were similar on fresh

and dry weight bases. At high  $\text{NO}_3$  levels the dry weight of plant produced was substantially less than that comparable with the fresh weight, indicating that high  $\text{NO}_3$  tends to produce a succulent plant. To a lesser extent the same is true of the dry weight of  $\text{NH}_4\text{-NO}_3$  grown plants. Difference in dry weights of roots between treatments were much less, in fact, the total difference between all treatments was slight, indicating that succulence is a condition of the overground parts.

Increases in per cent moisture content were manifested mainly in the leaves, where the moisture content varied between 82 and 87%. Plants having low water content matured earlier than the more succulent plants.

The ratio of leaf/stem (Table V) varied between 0.6 at low nitrate and 1.0 at high nitrate levels. The comparable ratios where  $\text{NH}_4\text{-NO}_3$  were used were all lower, indicating that nitrate tends to stimulate leaf development while ammonium ion tends to stimulate stem development. The pattern of leaf/root ratios followed the same direction as leaf/stem, but the magnitude was higher. At low nitrogen levels the plant synthesizes root in preference to leaf and stem tissue. The ratio of leaf to the total plant was so uniform as to indicate the belladonna plant is most strikingly responsive to nutritional treatments through its leaves. Where plants were furnished adequate nitrogen, i. e., 10 mg equiv. or more of either  $\text{NO}_3$  or  $\text{NH}_4\text{-NO}_3$ , the following decimal fractional parts constitute the belladonna plant:

	Fresh Weight	Dry Weight
Leaf	0.35	0.38
Stem	0.35	0.40
Flowers	0.05	0.04
Root	0.25	0.18

## DISCUSSION

The water culture method lends itself well to growth of the belladonna plant, where all nutritional variables can be controlled and growth can be measured periodically without sacrificing the plants.

These plants grew well in the greenhouse under such conditions to full maturity, within a period of fifty-five days, without aeration of roots, which compares favorably with the growth cycle of field-grown plants. They differed from field-grown plants mainly in that lateral branching occurred to a lesser degree. By contrast, branching discussed in this work involved branches formed at the shoot apex as a result of floral initiation, and elongation of these branches became inflorescences. This type of branching also occurs with field-grown plants.

After a short period of growth, uptake of ions by the plant caused the hydrogen ion concentration to change, for which adjustment to pH 5.8 was made weekly. The drift was invariably toward the alkaline range, due to preferential uptake of  $\text{NO}_3$ . During periods of rapid growth and salt uptake the solution pH shifted as high as to pH 7.5; but most adjustments involved not more than 1.5 pH units. Occasionally iron citrate was added in quantities greater than 1 ml. per week when incipient chlorosis indicated a need for iron.

Growth data were based on the means of 12 plants per treatment, allowing statistical support of differences where applicable.

TABLE IV.—NUMBER OF APICAL BRANCHES AND FLOWERS OF BELLADONNA PLANTS<sup>a</sup>

Solution Composition, mg. equiv. of N/L.	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Branches	2	2.8	3.9	4.2	3.5	4.4	2	4.7	7.9
Flowers	16.0	22.5	24.0	30.0	25.0	28.0	18.0	35.0	48.5

<sup>a</sup> Numbers are means of 12 plants.

TABLE V.—RATIOS (FRESH WEIGHT) OF PLANT PARTS OF THE BELLADONNA PLANT

Solution Composition, mg. equiv. of N/L.	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf/stem	0.61	0.76	0.86	0.96	1.00	1.01	0.61	0.96	0.97
Leaf/root	0.77	1.21	1.21	1.30	1.47	1.43	0.62	1.43	1.48
Leaf/total plant	0.24	0.29	0.31	0.34	0.36	0.36	0.23	0.33	0.33

Since  $\text{NO}_3$  must be reduced to  $\text{NH}_3$  and then to amino acids by amination within the plant tissue, it is to be expected that the  $\text{NH}_3$  source would shorten the enzymatically controlled synthetic process.

The number of expanded leaves increased only slightly with increased  $\text{NO}_3$ , indicating that it is the function of the organized meristem to cut off leaf initials at a fairly uniform rate, and that nutritional variables exert a far more pronounced effect on the rate of elongation of the produced initial than on its rate of production. At high levels of  $\text{NO}_3$  the normal floral initiation of shoot apex is curtailed and the vegetative condition is enhanced, thereby producing the well-known succulent condition in plants. The production of dry weight with  $\text{NH}_3\text{-NO}_3$  was significantly greater than that with  $\text{NO}_3$  alone, indicating that increased water in tissue did not occur. This would be due to the fact that the tissue does not accumulate  $\text{NH}_3$  in great excess over its metabolic needs as it does  $\text{NO}_3$ .

The ratio of leaf to root and stem to root under low nitrogen supply point to the larger root system developed under these conditions. Ammonia- $\text{NO}_3$  at adequate levels appears to stimulate stem development as indicated by leaf to stem and stem to root ratios.

## SUMMARY

1. Belladonna plants were grown from seed to a selected stage of flower maturity for each of a series of nutritional treatments in water culture over a range of  $\text{NO}_3$  and  $\text{NH}_3\text{-NO}_3$  sources with all of the other known essential elements present at a constant adequate level.

2. Twelve plants were grown to a selected stage of flower maturity for each nutritional treatment which furnished sufficient experimental material to replicate satisfactorily all of the chemical tests that were performed.

3. Growth was characterized by the effect of these variables on shoot length, fresh and dry weight of all plant parts, degree of branching, leaf initiation, flower formation, and the ratio of plant parts to each other.

4. By most of the criteria observed, growth of the plant was better with the  $\text{NH}_3\text{-NO}_3$  source than with  $\text{NO}_3$  alone. On the basis of dry weight produced, the most satisfactory growth was obtained from  $\text{NO}_3$  at 20 mg. equiv. and from  $\text{NH}_3\text{-NO}_3$  at 30 mg. equiv.

5. High levels of  $\text{NO}_3$  tend to raise the tissue water level, producing a more succulent plant than that furnished  $\text{NH}_3\text{-NO}_3$ . Because of this variation the dry weight is a more reliable criterion for assessing growth changes.

6. The root system was unusually large under low levels of nitrogen.

## REFERENCES

- (1) James, W. O., *et al.*, "Oxford Medicinal Plants Scheme," Annual Reports 1942, 1943, 1944, 1945.
- (2) James, W. O., *Nature*, 158, 654 (1946).
- (3) James, W. O., *ibid.*, 159, 196 (1947).
- (4) James, G. M., *Econ. Botany*, 1, 230 (1947).
- (5) Brewer, W. R., and Hiner, L. D., *This Journal*, 39, 586 (1950).
- (6) Prasad, S., *ibid.*, 36, 180 (1947).
- (7) Ahmed, Z. F., and Fahmy, I. R., *ibid.*, 38, 484 (1949).
- (8) Hoagland, D. R., and Arnon, D. I., *Calif. Agr. Expt. Sta. Circular 347*, Berkeley, Rev. 1950.

# Nitrogenous Constituents of *Atropa belladonna* L. Grown on Different Sources of Externally Supplied Nitrogen\*

By L. J. SCHERMEISTER†, F. A. CRANE, and R. F. VOIGT

Nitrates, nitrites, ammonia, amides, amino acids, proteins, and alkaloids were determined in plants under all treatments as previously described. At low nitrogen supply, belladonna synthesized alkaloids preferentially over protein. At high levels more protein and soluble nitrogenous compounds accumulated in tissue while the alkaloid level was relatively low. There appeared to be no direct relation between alkaloid and protein.  $\gamma$ -Aminobutyric acid may be a precursor to the belladonna alkaloids based on amounts of each present.

PLANTS grown by methods explained in the previous report (1) were harvested and prepared for the determination of the various nitrogenous constituents mentioned below. Samples were taken that would provide fresh and dry weights of each plant part, total nitrogen, nitrate, ammonia, amides, amino acids both free and combined in protein, and alkaloids of the various parts.

## PROCEDURE

Samples for total nitrogen (Kjeldahl) were taken directly from fresh tissue to avoid nitrogen loss on drying. Tissues used for the determination of nitrate, nitrite, ammonia, and amides were frozen at  $-20^{\circ}$  from the fresh plant at the time of harvest. Samples used for amino acid determination were placed in sufficient 95% ethyl alcohol that with the water normally present in the tissue resulted in a 75% alcohol concentration. If more alcohol was needed to cover tissue, 75% alcohol was added. The remaining tissue was dried for alkaloid determinations.

Ammonia, amide, nitrite, and nitrate fractions were determined by the method of Varner, Bulen, Vanecko, and Burrell (2) which separates these fractions by a combination of Dowex resins and selective pH ranges. This releases the fractions individually so that they can be distilled and titrated in a semimicroKjeldahl apparatus.

Free amino acids and those combined in protein (after acid hydrolysis) were determined by paper chromatography made quantitative by Thompson and Steward (3). Washed and buffered (pH 7.0) sheets of Whatman No. 1 filter papers were used, making reproducibility such that multiple determinations were never more than 5% from each other.

Alkaloids were determined by the method of Brown, Kireh, and Webster (4) with the following variations: (a) a 3-Gm rather than a 10-Gm sample was employed, (b) the material was extracted with

3 ml of stronger ammonia test solution U S P and 6 ml of ether, U S P, instead of 10 ml and 20 ml, respectively, (c) the total extractive was employed in the chromatographic separation of the alkaloid, instead of a 10-ml aliquot, (d) the total alkaloids were titrated electrometrically to the end point of pH 6.5 instead of using the methyl red indicator.

Total nitrogen in each tissue was determined by the Kjeldahl method described in the U S P XIV (5), including the following modifications: (a) A 1-Gm sample of fresh tissue containing portions from exactly the same areas (apical, median, and basal) of the respective plant parts at the same stage of development were placed in the sulfuric acid digesting mixture and quantitatively transferred into semimicroKjeldahl digestion flasks by rinsing with distilled water. (b) The rinse water was evaporated to approximately 5 ml over a Bunsen burner. After the digestion flask had cooled to room temperature, 0.5 ml of 30% hydrogen peroxide was added. The sample was digested until the solution acquired a clear bluish green color. (c) To absorb heat produced upon addition of sodium hydroxide, the digestion flask was immersed in a cold water bath. (d) Fifteen milliliters of 10 N sodium hydroxide containing two drops of methyl red indicator was cautiously added, while a few bubbles of steam were coming over from the generator, insuring immediate mixing of the sulfuric acid and the sodium hydroxide. When all of the sodium hydroxide had been added, distillation with steam was begun at once. (e) Ten drops of the indicator used by Ma and Zuazaga (6) and 25 ml of 2% boric acid were placed in the receiving flask.

The ammonia collected in the receiving flask was titrated according to the official U S P method and total nitrogen calculated.

## RESULTS

### Nitrogenous Constituents

**Total Nitrogen.**—The belladonna plant follows the commonly reported pattern of absorbing nitrogen (Tables I and II) from a source far in excess of its growth requirements. The greatest absorption was in tissues furnished nitrate (23 mg nitrogen per Gm of fresh tissue), but since the  $\text{NH}_4\text{-NO}_3$  plants grew more vigorously, the amount of nitrogen per total plant exceeded that of the plants furnished

\* Received September 25, 1959, from the University of Illinois College of Pharmacy, Chicago 12.

This paper is based on a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree Doctor of Philosophy.

† Present address: School of Pharmacy, North Dakota Agricultural College, Fargo.

TABLE I.—TOTAL NITROGEN OF BELLADONNA PLANTS (KJELDAHL)<sup>a</sup>

	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	3.98	4.15	4.40	5.63	6.60	5.50	3.10	4.67	5.77
Stem	1.37	2.10	2.14	2.58	3.14	3.02	0.92	3.17	3.56
Root	1.72	2.43	2.48	2.75	3.10	3.32	1.57	2.66	2.73
Flowers	5.40	6.75	7.06	7.26	8.00	10.10	5.34	7.05	6.77

<sup>a</sup> Figures are mg. total nitrogen/Gm. of tissue, fresh weight.TABLE II.—ABSOLUTE AMOUNTS OF TOTAL NITROGEN<sup>a</sup>

	Nitrate						Ammonia-Nitrate		
	5	10	15	20	25	30	50	20	30
Leaf	81.0	133.2	174.2	243.0	316.8	285.4	58.9	241.0	335.7
Stem	46.0	90.3	98.7	119.2	146.3	144.9	28.6	176.9	217.8
Root	46.1	66.1	82.1	92.2	103.8	120.0	47.3	99.1	109.5
Flower	24.8	39.8	47.3	55.1	52.8	57.6	26.2	64.6	87.1
Total plant	197.9	329.4	402.3	509.5	619.7	607.9	161.0	581.6	750.1

<sup>a</sup> Figures are mg. total nitrogen per plant part.TABLE III.—NITRATE, NITRITE, AMMONIA, AND AMIDE NITROGEN OF BELLADONNA PLANTS AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Solu. Compn., mg. Equiv. Nitrogen/L.		Nitrate						Ammonia-Nitrate		
		5	10	15	20	25	30	5	20	30
Nitrate determined in tissue	Leaf	0.031	0.030	0.042	0.071	0.195	0.274	0.031	0.050	0.064
	Stem	0.030	0.050	0.166	0.280	0.257	0.267	0.032	0.121	0.055
	Root	0.030	0.045	0.050	0.077	0.152	0.162	0.032	0.048	0.062
Nitrite determined in tissue	Leaf	...	...	...	...	...	...	...	...	...
	Stem	...	...	...	...	...	...	...	...	0.03
	Root	...	...	...	...	...	...	...	...	0.03
Ammonia determined in tissue	Leaf	...	...	...	0.015	0.016	0.018	...	0.013	0.018
	Stem	...	0.063	0.073	0.124	0.182	0.184	...	0.184	0.218
	Root	...	...	...	0.020	0.025	0.030	...	0.023	0.025
Amide determined in tissue	Leaf	0.034	0.059	0.061	0.070	0.071	0.064	0.041	0.070	0.080
	Stem	0.034	0.122	0.176	0.284	0.315	0.410	0.046	0.311	0.382
	Root	0.036	0.026	0.039	0.050	0.062	0.090	0.029	0.028	0.032

<sup>a</sup> Nitrogen, mg./Gm. fresh weight.

nitrate alone. Flower tissue contained the highest concentration of total nitrogen on a "per Gm. of tissue" basis but the amount of tissue was small.

**Nitrate and Ammonia-Nitrate.**—These were absorbed from solutions by the plants at all levels (Table III). At the lower levels of nitrate and at all levels of  $\text{NH}_3\text{-NO}_3$  the order of absorption indicates its direct use without accumulation in the tissue. At higher  $\text{NO}_3$  levels there was clear evidence of accumulation in the tissue far beyond the ability of the plant to metabolize it.

**Nitrite.**—Although nitrites in relatively small concentrations are considered toxic and do not generally accumulate in plants, detectable amounts (0.03 mg./Gm.) did occur in stem and root tissues of belladonna at 30 mg./equiv. of  $\text{NH}_3\text{-NO}_3$ . This can be interpreted as an inability of the plant to convert readily all the available nitrate into ammonia via nitrite because of the concentration of ammonia already present in these tissues.

**Ammonia Nitrogen.**—Detected chiefly in stems at the higher levels of nitrate and especially  $\text{NH}_3\text{-NO}_3$  supply, this indicates that ammonium is commonly utilized in tissue so rapidly that it is detected only in the area of translocation.

**Amides.**—Like ammonia, these are metabolized quickly in leaf and root and are present in an appreciable quantity in stem. The presence of the

major amides was detected along with the amino acids, chromatographically.

**Amino Acids and Amides.**—Those present in the alcohol-soluble fraction (free amino acids) (Tables IV, V, and VI) include cysteine acid, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, proline, valine, the leucines, and  $\gamma$ -aminobutyric acid. Associated with increasing nitrate supply are greatly increased amounts of aspartic acid, glutamic acid, glutamine, and proline. Plants grown at the higher  $\text{NH}_3\text{-NO}_3$  levels contained 50% more soluble nitrogen in the leaf (Table IV) than the corresponding  $\text{NO}_3$  examples, the difference being made up of larger quantities of glutamic acid, glutamine, proline, and the presence of significant quantities of asparagine that were not present in the nitrate grown plants.

It is noteworthy that glutamine as a storage form of nitrogen increased up to 25 mg. equivalents of  $\text{NO}_3$ . Above this level a greater proportion of the nitrogen was converted into protein.

Asparagine, which accumulated at the upper levels of ammonium was not present at any level of nitrate supply. This bears out the idea of Steward and Preston (7) that glutamine is intimately associated with nitrogen synthesis and asparagine is associated with protein hydrolysis in the tissue.

It is surprising that a closed ring heterocyclic

# Nitrogenous Constituents of *Atropa belladonna* L. Grown on Different Sources of Externally Supplied Nitrogen\*

By L. J. SCHIERMEISTER†, F. A. CRANE, and R. F. VOIGT

Nitrates, nitrites, ammonia, amides, amino acids, proteins, and alkaloids were determined in plants under all treatments as previously described. At low nitrogen supply, belladonna synthesized alkaloids preferentially over protein. At high levels more protein and soluble nitrogenous compounds accumulated in tissue while the alkaloid level was relatively low. There appeared to be no direct relation between alkaloid and protein.  $\gamma$ -Aminobutyric acid may be a precursor to the belladonna alkaloids based on amounts of each present.

PLANTS grown by methods explained in the previous report (1) were harvested and prepared for the determination of the various nitrogenous constituents mentioned below. Samples were taken that would provide fresh and dry weights of each plant part, total nitrogen, nitrate, ammonia, amides, amino acids both free and combined in protein, and alkaloids of the various parts.

## PROCEDURE

Samples for total nitrogen (Kjeldahl) were taken directly from fresh tissue to avoid nitrogen loss on drying. Tissues used for the determination of nitrate, nitrite, ammonia, and amides were frozen at  $-20^{\circ}$  from the fresh plant at the time of harvest. Samples used for amino acid determination were placed in sufficient 95% ethyl alcohol that with the water normally present in the tissue resulted in a 75% alcohol concentration. If more alcohol was needed to cover tissue, 75% alcohol was added. The remaining tissue was dried for alkaloid determinations.

Ammonia, amide, nitrite, and nitrate fractions were determined by the method of Varner, Bulen, Vanecko, and Burrell (2) which separates these fractions by a combination of Dowex resins and selective pH ranges. This releases the fractions individually so that they can be distilled and titrated in a semimicroKjeldahl apparatus.

Free amino acids and those combined in protein (after acid hydrolysis) were determined by paper chromatography made quantitative by Thompson and Steward (3). Washed and buffered (pH 7.0) sheets of Whatman No. 1 filter papers were used, making reproducibility such that multiple determinations were never more than 5% from each other.

Alkaloids were determined by the method of Brown, Kirch, and Webster (4) with the following variations: (a) a 3-Gm. rather than a 10-Gm. sample was employed; (b) the material was extracted with

3 ml. of stronger ammonia test solution U. S. P. and 6 ml. of ether, U. S. P., instead of 10 ml. and 20 ml., respectively; (c) the total extractive was employed in the chromatographic separation of the alkaloid, instead of a 10-ml. aliquot; (d) the total alkaloid were titrated electrometrically to the end point of pH 6.5 instead of using the methyl red indicator.

Total nitrogen in each tissue was determined by the Kjeldahl method described in the U. S. P. XIV (5), including the following modifications: (a) A 1-Gm. sample of fresh tissue containing portions from exactly the same areas (apical, median, and basal) of the respective plant parts at the same stage of development were placed in the sulfuric acid digesting mixture and quantitatively transferred into semimicroKjeldahl digestion flasks by rinsing with distilled water. (b) The rinse water was evaporated to approximately 5 ml. over a Bunsen burner. After the digestion flask had cooled to room temperature, 0.5 ml. of 30% hydrogen peroxide was added. The sample was digested until the solution acquired a clear bluish green color. (c) To absorb heat produced upon addition of sodium hydroxide, the digestion flask was immersed in a cold water bath. (d) Fifteen milliliters of 10 N sodium hydroxide containing two drops of methyl red indicator was cautiously added, while a few bubbles of steam were coming over from the generator, insuring immediate mixing of the sulfuric acid and the sodium hydroxide. When all of the sodium hydroxide had been added, distillation with steam was begun at once. (e) Ten drops of the indicator used by Ma and Zuazaga (6) and 25 ml. of 2% boric acid were placed in the receiving flask.

The ammonia collected in the receiving flask was titrated according to the official U. S. P. method and total nitrogen calculated.

## RESULTS

### Nitrogenous Constituents

**Total Nitrogen.**—The belladonna plant follows the commonly reported pattern of absorbing nitrogen (Tables I and II) from a source far in excess of its growth requirements. The greatest absorption was in tissues furnished nitrate (23 mg. nitrogen per Gm. of fresh tissue), but since the  $\text{NH}_4\text{NO}_3$  plants grew more vigorously, the amount of nitrogen per total plant exceeded that of the plants furnished

\* Received September 25, 1959, from the University of Illinois, College of Pharmacy, Chicago 12.

† This paper is based on a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree Doctor of Philosophy.

† Present address: School of Pharmacy, North Dakota Agricultural College, Fargo.

TABLE I.—TOTAL NITROGEN OF BELLADONNA PLANTS (KJELDAHL)<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	3 98	4 15	4 40	5 63	6 60	5 50	3 10	4 67	5 77
Stem	1 37	2 10	2 14	2 58	3 14	3 02	0 92	3 17	3 56
Root	1.72	2 43	2 48	2 75	3 10	3 32	1 57	2 66	2 73
Flowers	5 40	6 75	7 06	7 26	8 00	10 10	5 34	7 05	6 77

<sup>a</sup> Figures are mg total nitrogen/Gm of tissue, fresh weightTABLE II —ABSOLUTE AMOUNTS OF TOTAL NITROGEN<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	81 0	133 2	174 2	243 0	316 8	285 4	58.9	241 0	335 7
Stem	46 0	90 3	98 7	119 2	146 3	144 9	28 6	176 9	217 8
Root	46 1	66 1	82 1	92 2	103 8	120 0	47 3	99 1	109 5
Flower	24 8	39 8	47 3	55 1	52 8	57 6	26 2	64 6	87 1
Total plant	197 9	329 4	402 3	509 5	619 7	607 9	161 0	581 6	750 1

<sup>a</sup> Figures are mg total nitrogen per plant partTABLE III —NITRATE, NITRITE, AMMONIA, AND AMIDE NITROGEN OF BELLADONNA PLANTS AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Solu Compn , mg Equiv Nitrogen/L			Nitrate						Ammonia Nitrate		
			5	10	15	20	25	30	5	20	30
Nitrate determined in tissue		Leaf	0 031	0 030	0 042	0 071	0 195	0 274	0 031	0 050	0 064
		Stem	0 030	0 050	0 166	0 280	0 257	0 267	0 032	0 121	0 055
		Root	0.030	0 045	0 050	0 077	0 152	0 162	0 032	0 048	0 062
Nitrite determined in tissue		Leaf									0 03
		Stem									0 03
		Root									0 03
Ammonia determined in tissue		Leaf				0 015	0 016	0 018		0 013	0 018
		Stem		0 063	0 073	0 124	0 182	0 184		0 184	0 218
		Root				0 020	0 025	0 030		0 023	0 025
Amide determined in tissue		Leaf	0 034	0 059	0 061	0 070	0 071	0 064	0 041	0 070	0 080
		Stem	0 034	0 122	0 176	0 284	0 315	0 410	0 046	0 311	0 382
		Root	0 036	0 026	0 039	0 050	0 062	0 090	0 029	0 028	0 032

<sup>a</sup> Nitrogen, mg /Gm fresh weight

nitrate alone. Flower tissue contained the highest concentration of total nitrogen on a "per Gm of tissue" basis but the amount of tissue was small.

**Nitrate and Ammonia-Nitrate.**—These were absorbed from solutions by the plants at all levels (Table III). At the lower levels of nitrate and at all levels of  $\text{NH}_3\text{-NO}_3$  the order of absorption indicates its direct use without accumulation in the tissue. At higher  $\text{NO}_3$  levels there was clear evidence of accumulation in the tissue far beyond the ability of the plant to metabolize it.

**Nitrite.**—Although nitrites in relatively small concentrations are considered toxic and do not generally accumulate in plants, detectable amounts (0.03 mg /Gm) did occur in stem and root tissues of belladonna at 30 mg /equiv of  $\text{NH}_3\text{-NO}_3$ . This can be interpreted as an inability of the plant to convert readily all the available nitrate into ammonia via nitrite because of the concentration of ammonia already present in these tissues.

**Ammonia Nitrogen.**—Detected chiefly in stems at the higher levels of nitrate and especially  $\text{NH}_3\text{-NO}_3$  supply, this indicates that ammonium is commonly utilized in tissue so rapidly that it is detected only in the area of translocation.

**Amides.**—Like ammonia, these are metabolized quickly in leaf and root and are present in an appreciable quantity in stem. The presence of the

major amides was detected along with the ammonia, chromatographically.

**Amino Acids and Amides.**—Those present in the alcohol-soluble fraction (free amino acids) (Tables IV, V, and VI) include cysteine acid, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, proline, valine, the leucines, and  $\gamma$ -aminobutyric acid. Associated with increasing nitrate supply are greatly increased amounts of aspartic acid, glutamic acid, glutamine, and proline. Plants grown at the higher  $\text{NH}_3\text{-NO}_3$  levels contained 50% more soluble nitrogen in the leaf (Table IV) than the corresponding  $\text{NO}_3$  examples, the difference being made up of larger quantities of glutamic acid, glutamine, proline, and the presence of significant quantities of asparagine that were not present in the nitrate grown plants.

It is noteworthy that glutamine as a storage form of nitrogen increased up to 25 mg equivalents of  $\text{NO}_3$ . Above this level a greater proportion of the nitrogen was converted into protein.

Asparagine, which accumulated at the upper levels of ammonium was not present at any level of nitrate supply. This bears out the idea of Steward and Preston (7) that glutamine is intimately associated with nitrogen synthesis and asparagine is associated with protein hydrolysis in the tissue.

It is surprising that a closed ring heterocyclic

# Nitrogenous Constituents of *Atropa belladonna* L. Grown on Different Sources of Externally Supplied Nitrogen\*

By L. J. SCHIERMEISTER†, F. A. CRANE, and R. F. VOIGT

Nitrates, nitrites, ammonia, amides, amino acids, proteins, and alkaloids were determined in plants under all treatments as previously described. At low nitrogen supply, belladonna synthesized alkaloids preferentially over protein. At high levels more protein and soluble nitrogenous compounds accumulated in tissue while the alkaloid level was relatively low. There appeared to be no direct relation between alkaloid and protein.  $\gamma$ -Aminobutyric acid may be a precursor to the belladonna alkaloids based on amounts of each present.

PLANTS grown by methods explained in the previous report (1) were harvested and prepared for the determination of the various nitrogenous constituents mentioned below. Samples were taken that would provide fresh and dry weights of each plant part, total nitrogen, nitrate, ammonia, amides, amino acids both free and combined in protein, and alkaloids of the various parts.

## PROCEDURE

Samples for total nitrogen (Kjeldahl) were taken directly from fresh tissue to avoid nitrogen loss on drying. Tissues used for the determination of nitrate, nitrite, ammonia, and amides were frozen at  $-20^{\circ}$  from the fresh plant at the time of harvest. Samples used for amino acid determination were placed in sufficient 95% ethyl alcohol that with the water normally present in the tissue resulted in a 75% alcohol concentration. If more alcohol was needed to cover tissue, 75% alcohol was added. The remaining tissue was dried for alkaloid determinations.

Ammonia, amide, nitrite, and nitrate fractions were determined by the method of Varner, Inken, Vanecko, and Burrell (2) which separates these fractions by a combination of Dowex resins and selective pH ranges. This releases the fractions individually so that they can be distilled and titrated in a semimicroKjeldahl apparatus.

Free amino acids and those combined in protein (after acid hydrolysis) were determined by paper chromatography made quantitative by Thompson and Steward (3). Washed and buffered (pH 7.0) sheets of Whatman No. 1 filter papers were used, making reproducibility such that multiple determinations were never more than 5% from each other.

Alkaloids were determined by the method of Brown, Kirch, and Webster (4) with the following variations: (a) a 3-Gm. rather than a 10-Gm. sample was employed; (b) the material was extracted with

3 ml. of stronger ammonia test solution U. S. P. and 5 ml. of ether, U. S. P., instead of 10 ml. and 20 ml., respectively; (c) the total extractive was employed in the chromatographic separation of the alkaloid, instead of a 10-ml. aliquot; (d) the total alkaloids were titrated electrometrically to the end point of pH 6.5 instead of using the methyl red indicator.

Total nitrogen in each tissue was determined by the Kjeldahl method described in the U. S. P. XIV (5), including the following modifications: (a) A 1-Gm. sample of fresh tissue containing portions from exactly the same areas (apical, median, and basal) of the respective plant parts at the same stage of development were placed in the sulfuric acid digesting mixture and quantitatively transferred into semimicroKjeldahl digestion flasks by rinsing with distilled water. (b) The rinse water was evaporated to approximately 5 ml. over a Bunsen burner. After the digestion flask had cooled to room temperature, 0.5 ml. of 30% hydrogen peroxide was added. The sample was digested until the solution acquired a clear bluish green color. (c) To absorb heat produced upon addition of sodium hydroxide, the digestion flask was immersed in a cold water bath. (d) Fifteen milliliters of 10 N sodium hydroxide containing two drops of methyl red indicator was cautiously added, while a few bubbles of steam were coming over from the generator, insuring immediate mixing of the sulfuric acid and the sodium hydroxide. When all of the sodium hydroxide had been added, distillation with steam was begun at once. (e) Ten drops of the indicator used by Ma and Zuazaga (6) and 25 ml. of 2% boric acid were placed in the receiving flask.

The ammonia collected in the receiving flask was titrated according to the official U. S. P. method and total nitrogen calculated.

## RESULTS

### Nitrogenous Constituents

**Total Nitrogen.**—The belladonna plant follows the commonly reported pattern of absorbing nitrogen (Tables I and II) from a source far in excess of its growth requirements. The greatest absorption was in tissues furnished nitrate (23 mg. nitrogen per Gm. of fresh tissue), but since the  $\text{NH}_4\text{-NO}_3$  plants grew more vigorously, the amount of nitrogen per total plant exceeded that of the plants furnished

\* Received September 25, 1959, from the University of Illinois, College of Pharmacy, Chicago 12.

† This paper is based on a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree Doctor of Philosophy.

† Present address: School of Pharmacy, North Dakota Agricultural College, Fargo.

TABLE I—TOTAL NITROGEN OF BELLADONNA PLANTS (KJELOAHL)<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	3.98	4.15	4.40	5.63	6.60	5.50	3.10	4.67	5.77
Stem	1.37	2.10	2.14	2.58	3.14	3.02	0.92	3.17	3.56
Root	1.72	2.43	2.48	2.75	3.10	3.32	1.57	2.66	2.73
Flowers	5.40	6.75	7.06	7.26	8.00	10.10	5.34	7.05	6.77

<sup>a</sup> Figures are mg total nitrogen/Gm of tissue, fresh weightTABLE II—ABSOLUTE AMOUNTS OF TOTAL NITROGEN<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	50	20	30
Leaf	81.0	133.2	174.2	243.0	316.8	285.4	58.9	241.0	335.7
Stem	46.0	90.3	98.7	119.2	146.3	144.9	28.6	176.9	217.8
Root	46.1	66.1	82.1	92.2	103.8	120.0	47.3	99.1	109.5
Flower	24.8	39.8	47.3	55.1	52.8	57.6	26.2	64.6	87.1
Total plant	197.9	329.4	402.3	509.5	619.7	607.9	161.0	581.6	750.1

<sup>a</sup> Figures are mg total nitrogen per plant partTABLE III—NITRATE, NITRITE, AMMONIA, AND AMIDE NITROGEN OF BELLADONNA PLANTS AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Soln Compn, mg Equiv Nitrogen/L		Nitrate						Ammonia Nitrate		
		5	10	15	20	25	30	5	20	30
Nitrate determined in tissue	Leaf	0.031	0.030	0.042	0.071	0.195	0.274	0.031	0.050	0.064
	Stem	0.030	0.050	0.166	0.280	0.257	0.267	0.032	0.121	0.055
	Root	0.030	0.045	0.050	0.077	0.152	0.162	0.032	0.048	0.062
Nitrite determined in tissue	Leaf									
	Stem									0.03
	Root									0.03
Ammonia determined in tissue	Leaf				0.015	0.016	0.018		0.013	0.018
	Stem		0.063	0.073	0.124	0.182	0.184		0.184	0.218
	Root				0.020	0.025	0.030		0.023	0.025
Amide determined in tissue	Leaf	0.034	0.059	0.061	0.070	0.071	0.064	0.041	0.070	0.080
	Stem	0.034	0.122	0.176	0.284	0.315	0.410	0.046	0.311	0.382
	Root	0.036	0.026	0.039	0.050	0.062	0.090	0.029	0.028	0.032

<sup>a</sup> Nitrogen, mg /Gm fresh weight

nitrate alone. Flower tissue contained the highest concentration of total nitrogen on a "per Gm of tissue" basis but the amount of tissue was small.

**Nitrate and Ammonia-Nitrate.**—These were absorbed from solutions by the plants at all levels (Table III). At the lower levels of nitrate and at all levels of  $\text{NH}_3\text{-NO}_3$  the order of absorption indicates its direct use without accumulation in the tissue. At higher  $\text{NO}_3$  levels there was clear evidence of accumulation in the tissue far beyond the ability of the plant to metabolize it.

**Nitrite.**—Although nitrites in relatively small concentrations are considered toxic and do not generally accumulate in plants, detectable amounts (0.03 mg /Gm) did occur in stem and root tissues of belladonna at 30 mg /equiv of  $\text{NH}_3\text{-NO}_3$ . This can be interpreted as an inability of the plant to convert readily all the available nitrate into ammonia via nitrite because of the concentration of ammonia already present in these tissues.

**Ammonia Nitrogen.**—Detected chiefly in stems at the higher levels of nitrate and especially  $\text{NH}_3\text{-NO}_3$  supply, this indicates that ammonium is commonly utilized in tissue so rapidly that it is detected only in the area of translocation.

**Amides.**—Like ammonia, these are metabolized quickly in leaf and root and are present in an appreciable quantity in stem. The presence of the

major amides was detected along with the amino acids, chromatographically.

**Amino Acids and Amides.**—Those present in the alcohol-soluble fraction (free amino acids) (Tables IV, V, and VI) include cysteine acid, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, proline, valine, the leucines, and  $\gamma$ -amino butyric acid. Associated with increasing nitrate supply are greatly increased amounts of aspartic acid, glutamic acid, glutamine, and proline. Plants grown at the higher  $\text{NH}_3\text{-NO}_3$  levels contained 50% more soluble nitrogen in the leaf (Table IV) than the corresponding  $\text{NO}_3$  examples, the difference being made up of larger quantities of glutamic acid, glutamine, proline, and the presence of significant quantities of asparagine that were not present in the nitrate grown plants.

It is noteworthy that glutamine as a storage form of nitrogen increased up to 25 mg equivalents of  $\text{NO}_3$ . Above this level a greater proportion of the nitrogen was converted into protein.

Asparagine, which accumulated at the upper levels of ammonium was not present at any level of nitrate supply. This bears out the idea of Steward and Preston (7) that glutamine is intimately associated with nitrogen synthesis and asparagine is associated with protein hydrolysis in the tissue.

It is surprising that a closed ring heterocyclic



# Nitrogenous Constituents of *Atropa belladonna* L. Grown on Different Sources of Externally Supplied Nitrogen\*

By L. J. SCHERMEISTER†, F. A. CRANE, and R. F. VOIGT

Nitrates, nitrites, ammonia, amides, amino acids, proteins, and alkaloids were determined in plants under all treatments as previously described. At low nitrogen supply, belladonna synthesized alkaloids preferentially over protein. At high levels more protein and soluble nitrogenous compounds accumulated in tissue while the alkaloid level was relatively low. There appeared to be no direct relation between alkaloid and protein.  $\gamma$ -Aminobutyric acid may be a precursor to the belladonna alkaloids based on amounts of each present.

PLANTS grown by methods explained in the previous report (1) were harvested and prepared for the determination of the various nitrogenous constituents mentioned below. Samples were taken that would provide fresh and dry weights of each plant part, total nitrogen, nitrate, ammonia, amides, amino acids both free and combined in protein, and alkaloids of the various parts.

## PROCEDURE

Samples for total nitrogen (Kjeldahl) were taken directly from fresh tissue to avoid nitrogen loss on drying. Tissues used for the determination of nitrate, nitrite, ammonia, and amides were frozen at  $-20^{\circ}$  from the fresh plant at the time of harvest. Samples used for amino acid determination were placed in sufficient 95% ethyl alcohol that with the water normally present in the tissue resulted in a 75% alcohol concentration. If more alcohol was needed to cover tissue, 75% alcohol was added. The remaining tissue was dried for alkaloid determinations.

Ammonia, amide, nitrite, and nitrate fractions were determined by the method of Varner, Bulen, Vanecko, and Burrell (2) which separates these fractions by a combination of Dowex resins and selective pH ranges. This releases the fractions individually so that they can be distilled and titrated in a semimicroKjeldahl apparatus.

Free amino acids and those combined in protein (after acid hydrolysis) were determined by paper chromatography made quantitative by Thompson and Steward (3). Washed and buffered (pH 7.0) sheets of Whatman No. 1 filter papers were used, making reproducibility such that multiple determinations were never more than 5% from each other.

Alkaloids were determined by the method of Brown, Kireh, and Webster (4) with the following variations: (a) a 3-Gm rather than a 10 Gm sample was employed, (b) the material was extracted with

3 ml of stronger ammonia test solution U S P and 6 ml of ether, U S P, instead of 10 ml and 20 ml, respectively, (c) the total extractive was employed in the chromatographic separation of the alkaloid, instead of a 10-ml aliquot, (d) the total alkaloids were titrated electrometrically to the end point of pH 6.5 instead of using the methyl red indicator.

Total nitrogen in each tissue was determined by the Kjeldahl method described in the U S P XIV (5), including the following modifications: (a) A 1-Gm sample of fresh tissue containing portions from exactly the same areas (apical, median, and basal) of the respective plant parts at the same stage of development were placed in the sulfuric acid digesting mixture and quantitatively transferred into semimicroKjeldahl digestion flasks by rinsing with distilled water. (b) The rinse water was evaporated to approximately 5 ml over a Bunsen burner. After the digestion flask had cooled to room temperature, 0.5 ml of 30% hydrogen peroxide was added. The sample was digested until the solution acquired a clear bluish green color. (c) To absorb heat produced upon addition of sodium hydroxide, the digestion flask was immersed in a cold water bath. (d) Fifteen milliliters of 10 N sodium hydroxide containing two drops of methyl red indicator was cautiously added, while a few bubbles of steam were coming over from the generator, insuring immediate mixing of the sulfuric acid and the sodium hydroxide. When all of the sodium hydroxide had been added, distillation with steam was begun at once. (e) Ten drops of the indicator used by Ma and Zuazaga (6) and 25 ml of 2% boric acid were placed in the receiving flask.

The ammonia collected in the receiving flask was titrated according to the official U S P method and total nitrogen calculated.

## RESULTS

### Nitrogenous Constituents

**Total Nitrogen.**—The belladonna plant follows the commonly reported pattern of absorbing nitrogen (Tables I and II) from a source far in excess of its growth requirements. The greatest absorption was in tissues furnished nitrate (23 mg nitrogen per Gm of fresh tissue), but since the  $\text{NH}_3$ ,  $\text{NO}_3$  plants grew more vigorously, the amount of nitrogen per total plant exceeded that of the plants furnished

\* Received September 25, 1959 from the University of Illinois College of Pharmacy, Chicago 12.

This paper is based on a thesis submitted to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree Doctor of Philosophy.

† Present address: School of Pharmacy, North Dakota Agricultural College, Fargo.

TABLE I—TOTAL NITROGEN OF BELLADONNA PLANTS (KJELDAHL)<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	3.98	4.15	4.40	5.63	6.60	5.50	3.10	4.67	5.77
Stem	1.37	2.10	2.14	2.58	3.14	3.02	0.92	3.17	3.56
Root	1.72	2.43	2.48	2.75	3.10	3.32	1.57	2.66	2.73
Flowers	5.40	6.75	7.06	7.26	8.00	10.10	5.34	7.05	6.77

<sup>a</sup> Figures are mg total nitrogen/Gm of tissue, fresh weightTABLE II—ABSOLUTE AMOUNTS OF TOTAL NITROGEN<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	50	20	30
Leaf	81.0	133.2	174.2	243.0	316.8	285.4	58.9	241.0	335.7
Stem	46.0	90.3	98.7	119.2	146.3	144.9	28.6	176.9	217.8
Root	46.1	66.1	82.1	92.2	103.8	120.0	47.3	99.1	109.5
Flower	24.8	39.8	47.3	55.1	52.8	57.6	26.2	64.6	87.1
Total plant	197.9	329.4	402.3	509.5	619.7	607.9	161.0	581.6	750.1

<sup>a</sup> Figures are mg total nitrogen per plant partTABLE III—NITRATE, NITRITE, AMMONIA, AND AMIDE NITROGEN OF BELLADONNA PLANTS AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Soln. Compn, mg Equiv Nitrogen/L		Nitrate						Ammonia Nitrate		
		5	10	15	20	25	30	5	20	30
Nitrate determined in tissue	Leaf	0.031	0.030	0.042	0.071	0.195	0.274	0.031	0.050	0.064
	Stem	0.030	0.050	0.166	0.280	0.257	0.267	0.032	0.121	0.055
	Root	0.030	0.045	0.050	0.077	0.152	0.162	0.032	0.048	0.062
Nitrite determined in tissue	Leaf									
	Stem									0.03
	Root									0.03
Ammonia determined in tissue	Leaf				0.015	0.016	0.018		0.013	0.018
	Stem		0.063	0.073	0.124	0.182	0.184		0.184	0.218
	Root				0.020	0.025	0.030		0.023	0.025
Amide determined in tissue	Leaf	0.034	0.059	0.061	0.070	0.071	0.064	0.041	0.070	0.080
	Stem	0.034	0.122	0.176	0.284	0.315	0.410	0.046	0.311	0.382
	Root	0.036	0.026	0.039	0.050	0.062	0.090	0.029	0.028	0.032

<sup>a</sup> Nitrogen, mg/Gm fresh weight

nitrate alone. Flower tissue contained the highest concentration of total nitrogen on a "per Gm of tissue" basis but the amount of tissue was small.

**Nitrate and Ammonia-Nitrate.**—These were absorbed from solutions by the plants at all levels (Table III). At the lower levels of nitrate and at all levels of  $\text{NH}_3\text{-NO}_3$  the order of absorption indicates its direct use without accumulation in the tissue. At higher  $\text{NO}_3$  levels there was clear evidence of accumulation in the tissue far beyond the ability of the plant to metabolize it.

**Nitrite.**—Although nitrites in relatively small concentrations are considered toxic and do not generally accumulate in plants, detectable amounts (0.03 mg/Gm) did occur in stem and root tissues of belladonna at 30 mg/equiv of  $\text{NH}_3\text{-NO}_3$ . This can be interpreted as an inability of the plant to convert readily all the available nitrate into ammonia via nitrite because of the concentration of ammonia already present in these tissues.

**Ammonia Nitrogen.**—Detected chiefly in stems at the higher levels of nitrate and especially  $\text{NH}_3\text{-NO}_3$  supply, this indicates that ammonium is commonly utilized in tissue so rapidly that it is detected only in the area of translocation.

**Amides.**—Like ammonia, these are metabolized quickly in leaf and root and are present in an appreciable quantity in stem. The presence of the

major amides was detected along with the amino acids, chromatographically.

**Amino Acids and Amides.**—Those present in the alcohol-soluble fraction (free amino acids) (Tables IV, V, and VI) include cysteine acid, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, proline, valine, the leucines, and  $\gamma$ -aminobutyric acid. Associated with increasing nitrate supply are greatly increased amounts of aspartic acid, glutamic acid, glutamine, and proline. Plants grown at the higher  $\text{NH}_3\text{-NO}_3$  levels contained 50% more soluble nitrogen in the leaf (Table IV) than the corresponding  $\text{NO}_3$  examples, the difference being made up of larger quantities of glutamic acid, glutamine, proline, and the presence of significant quantities of asparagine that were not present in the nitrate grown plants.

It is noteworthy that glutamine as a storage form of nitrogen increased up to 25 mg equivalents of  $\text{NO}_3$ . Above this level a greater proportion of the nitrogen was converted into protein.

Asparagine, which accumulated at the upper levels of ammonium was not present at any level of nitrate supply. This bears out the idea of Steward and Preston (7) that glutamine is intimately associated with nitrogen synthesis and asparagine is associated with protein hydrolysis in the tissue.

It is surprising that a closed ring heterocyclic

TABLE IV —COMPOSITION OF THE ALCOHOL-SOLUBLE ORGANIC NITROGEN FRACTIONS OF BELLADONNA LEAF AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solutions, mg						Equiv./Liter		
	NO <sub>3</sub>						NO <sub>3</sub> NH <sub>4</sub>		
	5	10	15	20	25	30	5	20	30
Cysteine from cysteine		10 0	20 0	25 0	30 0	30 0		30 0	30 0
Aspartic	60 0	10 0	230 0	90 0	265 0	260 0	25 0	245.0	190 0
Glutamic	13 6	12 1	58 5	85 0	85 6	52 0	40 3	100 0	99 1
Serine	3 4		41 3	30 0	34 1	21 0	61 2	58 4	26 0
Glycine	14 1	23 6	10 7	21 6	31 9	46 5	13 0	62.9	61 2
Asparagine								130 0	150 0
Threonine		13 0	25 3	14 0	30 6	34 8	33 1	51 8	23 7
Alanine	47 5	70 7	55 2	76 3	113 3	53 4	70 0	175 2	142 6
Glutamine	34 0	37 5	284 6	375 0	472 5	270 0	110 0	1,375 0	655 0
Proline	333 0	531 6	600 0	644 0	1,010 0	789 0	133 0	1,147.0	1,554 0
Valine	14 7	18 9	27 9	17 5	20 3	11 1	23 3	35 5	23 1
Leucines	27 6	31 4	27 7	20 3	21 1	21 6	47 4	45 3	30 9
γ-Aminobutyric	72 2	104 5	161 5	150 0	167 3	90 1	150 8	305 0	200 0
Total, mcg /Gm	620 4	950 0	1,572 0	1,551 0	2,281 0	1,680 0	708 0	3,760 0	3,182 0

<sup>a</sup> Amino acid mcg /Gm fresh weightTABLE V —COMPOSITION OF THE ALCOHOL-SOLUBLE NITROGEN FRACTIONS OF BELLADONNA STEM AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solution mg						Equiv./Liter		
	NO <sub>3</sub>						NO <sub>3</sub> NH <sub>4</sub>		
	5	10	15	20	25	30	5	20	30
Cysteine from cysteine			25 0	40 0	35 0	35 0		34 0	37 0
Aspartic	90 0	92 0	130 0	200 0	190 0	210 0	100 0	160 0	290 0
Glutamic	28 4	31 2	34 2	66 0	53 1	51 2	12 0	53.9	36 4
Serine	14 2	28 0	22 9	35 7	26 0	38 2	10 1	33.9	19 3
Glycine	29 3	53 6	39 9	41 2	37 8	66 3	28 3	77.2	88 2
Asparagine	112 0	382 0	682 0	814 0	786 0	838 0		505 0	850 0
Threonine	9 2	46 8	28 8	58 3	45 5	44 5	19 8	64 1	60 5
Alanine	22 5	35 6	17 4	35 9	36 8	24 5	15.6	25 3	25 3
Glutamine	245 0	2,141 0	3,606 0	3,080 0	3,187 0	3,025 0	240 7	5,300 0	8,905 0
Proline	692 0	1,055 0	757 0	940 0	1,082 0	1,212 0	717 0	890 0	1,200 0
Valine	29 7	32 2	23 4	19 1	30 0	33 5	50 5	38 5	40 9
Leucines	64 3	131 7	47 2	45 3	46 7	70 4	83 9	84.5	87 1
Phenylalanine	65 0	177 0	71 8	63 5	77 5	100 7	58 5	63 6	65 6
Tyrosine	45 4	91 5	15 1	25 5	33 3	29 9	48 1	31 1	31 3
β-Alanine			22 2	34 1	30 0	20 0		15 8	17 0
γ-Aminobutyric	55 3	58 4	42 5	59 7	64 0	71 4	54 3	82 8	59 7
Total, mcg /Gm	1,501 0	4,356 0	5,467 0	5,556 0	5,760 0	5,870 0	1,438 0	7,459 0	11,813 0

<sup>a</sup> Amino acid, mcg /Gm fresh weightTABLE VI —COMPOSITION OF THE ALCOHOL-SOLUBLE ORGANIC NITROGEN FRACTIONS OF BELLADONNA ROOT AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solutions, mg						Equiv./Liter		
	NO <sub>3</sub>						NO <sub>3</sub> NH <sub>4</sub>		
	5	10	15	20	25	30	5	20	30
Aspartic	52 3	51 8	80 4	70 6	170 6	286 3	33 3	243 0	230 0
Glutamic	3 0	28 7	28 2	83 6	133 6	77 3	2 2	140 0	222.0
Serine		4 4	9 4	20 9	16 2	24 3		27 5	28 3
Glycine	6 0	16 7	7 9	24 2	29 7	37 1		58 0	112 9
Asparagine								84 0	535 0
Threonine		5 2	10 5	12 8	15 2	33 1		29 5	24 5
Alanine	3 4	13 3	10 6	33 1	36 1	83 0	4 6	56 9	50 2
Glutamine	41 6	43 6	30 0	45 2	102 2	131 0	54 0	2,676 0	5,002 0
Proline					172 3	230 9		172 0	390 0
Valine	4 6	14 7	7 4	7 1	22 1	14 6	2 9	16 0	16.9
Leucines		13 0	10 3	7 7	13 1	10 1	6 5	18 6	23.1
Phenylalanine		15 5		8 3	9 2	8 8	8 9	27 7	
γ-Aminobutyric	21 0	189 0	21 2	120 4	56 8	183 4	16 2	222 0	163 0
Total mcg /Gm	131 9	396 4	215 9	433 9	777 1	1,119.9	128 6	3,771 0	6,798 0

<sup>a</sup> Amino acid mcg /Gm fresh weight

amino acid such as proline should have constituted over 40% of the soluble nitrogen at all levels

The total soluble nitrogen produced was greater at each level of ammonia-nitrate than with nitrate-nitrogen alone. The major portion of the increase was due to the amides, asparagine, and glutamine at the higher nitrogen levels. The appearance of asparagine at the higher levels of ammonia-nitrate was accounted for by a reduction in the amount of aspartic acid in this series when compared with that supplied with nitrate. The combination of glutamic acid and glutamine under ammonia nutrition was more than three times that under the comparable nitrate nutrition, whereas the combination of aspartic acid and asparagine was roughly the same under both treatments.

The total amount of soluble nitrogen present in the stem (Table V) was approximately three times that found in the leaf. Aspartic and glutamic acids increased in the same proportions throughout the treatments, i.e., the aspartic acid was about three times the glutamic acid in most cases. Their amides also increased throughout the treatments but by contrast, glutamine was about three times the asparagine in all cases.

Phenylalanine, tyrosine,  $\beta$ -alanine were not detected in leaf tissue but were present in quantitative amounts in the stem.

The total amount of soluble nitrogen present in the roots (Table VI) that had been furnished nitrate was only half that present in the leaf tissue, but roots furnished the high level of  $\text{NH}_3\text{-NO}_3$  contained more than twice the soluble nitrogen of their leaves. This large amount of soluble nitrogen was predominantly asparagine and glutamine, indicating that the ammonia source stimulated the accumulation of amide in root tissue.  $\gamma$ -Aminobutyric acid is a major fraction in root tissue, particularly at the high levels of both  $\text{NO}_3$  and  $\text{NH}_3\text{-NO}_3$ . It was observed that an abundance of  $\gamma$ -aminobutyric acid seemed to be associated with

the presence of large amounts of alkaloid in the root.

**Alcohol-Insoluble Nitrogen (Protein).**—Although the nutrient supply had a pronounced effect on the metabolism of all plant parts, relatively slight changes in the amino acid composition of their protein was observed (Compare Tables IV and VII, V and VIII, VI and IX.) All of the amino acids present in the soluble fraction except  $\gamma$ -aminobutyric acid occurred much more abundantly in the protein than in the free state. The ratio of aspartic acid to glutamic acid in the leaf protein varied from 2:1 to 3:1 with the greater ratio being associated with the higher nitrogen levels. Serine, glycine, threonine, alanine, valine, phenylalanine, and tyrosine remained substantially constant throughout all of the treatments. All levels of ammonia-nitrate produced more protein than the corresponding nitrate treatment. Lysine and arginine commonly found in the protein fraction of plants were not detected in either the alcohol-soluble or insoluble fractions of the belladonna plant.

The composition of the stem protein (Table VIII) was similar to that of the leaf protein, but amounted to only one-tenth that of the leaf except at lowest  $\text{NO}_3$  levels. The ratio of aspartic acid to glutamic acid in the  $\text{NO}_3$  series was 2:1; in the  $\text{NH}_3\text{-NO}_3$  it was 1:1, indicating that  $\text{NH}_3$  tends to stimulate the production of glutamic acid over aspartic acid.

The protein synthesized in the root (Table IX) contained the compounds previously mentioned in leaf and stem and contained, in addition, cysteic acid in all treatments. It is surprising that this is the only sulfur-containing amino acid found in the protein fraction. The amount of protein synthesized in roots was about one-fourth that present in leaf and slightly more than twice that in stem. The compounds most noticeable in the root protein were the leucines, tyrosine, proline, and hydroxyproline. Of these, hydroxyproline is present in greater quantity than in any other plant part.

TABLE VII.—COMPOSITION OF THE ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF *Atropa belladonna* LEAF AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solution, mg. Equiv. /Liter						$\text{NO}_3\text{ NH}_3$		
	5	10	15	20	25	30	5	20	30
Cysteic from cysteine									
Aspartic	2,140	3,000	5,652	2,601	5,690	6,584	5,242	3,536	3,905
Glutamic	970	1,000	2,314	1,910	1,993	1,960	2,065	1,852	2,009
Serine	185	800	1,291	296	998	834	974	1,310	1,988
Glycine	430	700	1,045	679	882	919	550	596	1,865
Asparagine									
Threonine	910	1,100	1,394	1,240	1,159	1,100	1,099	1,352	1,606
Alanine	648	1,050	1,842	2,125	2,170	1,439	1,077	2,909	2,046
Glutamine									
Proline	730	1,200	2,883	1,425	3,000	3,952	1,319	4,660	4,470
Valine	1,175	1,425	1,833	1,668	1,880	2,100	1,391	2,490	2,625
Leucines	2,310	2,700	3,766	3,070	4,067	4,645	3,312	6,577	5,551
Phenylalanine	1,010	1,220	1,428	1,363	1,388	1,440	..	1,979	2,139
Tyrosine	747	570	553	503	631	744	690	930	695
$\beta$ -Alanine	..								
$\gamma$ -Aminobutyric									
OH Proline			266	175	247	184	276	552	423
Total mcg./Gm	11,355	14,765	24,267	17,055	24,105	25,900	17,995	28,743	29,322

<sup>a</sup> Amino acid, mcg./Gm fresh weight.

TABLE IV — COMPOSITION OF THE ALCOHOL-SOLUBLE ORGANIC NITROGEN FRACTIONS OF BELLADONNA LEAF AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solutions, mg Equiv /Liter						NO <sub>2</sub> NH <sub>2</sub>		
	5	10	15	20	25	30	5	20	30
Cysteic from cysteine		10 0	20 0	25 0	30 0	30 0		30 0	30 0
Aspartic	60 0	40 0	230 0	90 0	265 0	260 0	25 0	245 0	190 0
Glutamic	13 6	12 4	58 5	85 0	85 6	52 0	40 3	100 0	99 1
Serine	3 4		41 3	30 0	34 1	21 9	61 2	58 4	26 0
Glycine	14 4	23 6	10 7	24 6	31 9	46 5	13 0	62 9	61 2
Asparagine								130 0	150 0
Threonine		43 0	25 3	14 0	30 6	34 8	33 1	51 8	23 7
Alanine	47 5	70 7	55 2	76 3	113 3	53 4	70 0	175 2	142 6
Glutamine	34 0	37 5	284 6	375 0	472 5	270 0	110 0	1,375 0	655 0
Proline	333 0	531 6	600 0	644 0	1,010 0	789 0	133 0	1,147 0	1,554 0
Valine	14 7	18 9	27 9	17 5	20 3	11 4	23 3	35 5	23 1
Leucines	27 6	31 4	27 7	20 3	21 1	21 6	47 4	45 3	30 9
γ-Aminobutyric	72 2	104 5	161 5	150 0	167 3	90 1	150 8	305 0	200 0
Total, mcg /Gm	620 4	950 0	1 572 0	1,551 0	2,281 0	1,680 0	708 0	3,760 0	3,182 0

<sup>a</sup> Amino acid, mcg /Gm fresh weightTABLE V — COMPOSITION OF THE ALCOHOL-SOLUBLE NITROGEN FRACTIONS OF BELLADONNA STEM AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solution mg Equiv /Liter						NO <sub>2</sub> NH <sub>2</sub>		
	5	10	15	20	25	30	5	20	30
Cysteic from cysteine			25 0	40 0	35 0	35 0		34 0	37 0
Aspartic	90 0	92 0	130 0	200 0	190 0	210 0	100 0	160 0	290 0
Glutamic	28 4	31 2	34 2	66 0	53 1	51 2	12 0	53 9	36 4
Serine	14 2	28 0	22 9	35 7	26 0	38 2	10 1	33 9	19 3
Glycine	29 3	53 6	39 9	41 2	37 8	66 3	28 3	77 2	88 2
Asparagine	112 0	382 0	682 0	814 0	786 0	838 0		505 0	850 0
Threonine	9 2	46 8	28 8	58 3	45 5	44 5	19 8	64 1	60 5
Alanine	22 5	35 6	17 4	35 9	36 8	24 5	15 6	25 3	25 3
Glutamine	245 0	2,141 0	3,606 0	3,080 0	3,187 0	3,025 0	240 7	5,300 0	8,905 0
Proline	692 0	1,055 0	757 0	940 0	1,082 0	1,212 0	717 0	890 0	1,200 0
Valine	20 7	32 2	23 4	19 1	30 0	33 5	50 5	38 5	40 9
Leucines	64 3	131 7	47 2	45 3	46 7	70 4	83 9	84 5	87 1
Phenylalanine	65 0	177 0	71 8	63 5	77 5	100 7	58 5	63 6	65 6
Tyrosine	45 4	91 5	15 1	25 5	33 3	29 9	48 1	31 1	31 3
β-Alanine			22 2	34 1	30 0	20 0		15 8	17 0
γ-Aminobutyric	55 3	58 4	42 5	59 7	64 0	71 4	54 3	82 8	59 7
Total, mcg /Gm	1,501 0	4,356 0	5,467 0	5,556 0	5,760 0	5,870 0	1,438 0	7,459 0	11,813 0

<sup>a</sup> Amino acid mcg /Gm fresh weightTABLE VI — COMPOSITION OF THE ALCOHOL-SOLUBLE ORGANIC NITROGEN FRACTIONS OF BELLADONNA ROOT AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solutions, mg Equiv /Liter						NO <sub>2</sub> NH <sub>2</sub>		
	5	10	15	20	25	30	5	20	30
Aspartic	52 3	51 8	80 4	70 6	170 6	286 3	33 3	243 0	230 0
Glutamic	3 0	28 7	28 2	83 6	133 6	77 3	2 2	140 0	222 0
Serine		4 4	9 4	20 9	16 2	24 3		27 5	28 3
Glycine	6 0	16 7	7 9	24 2	29 7	37 1		58 0	112 9
Asparagine								84 0	535 0
Threonine		5 2	10 5	12 8	15 2	33 1		29 5	24 5
Alanine	3 4	13 3	10 6	33 1	36 1	83 0	4 6	56 9	50 2
Glutamine	41 6	43 6	30 0	45 2	102 2	131 0	54 0	2,676 0	5,002 0
Proline					172 3	230 9		172 0	390 0
Valine	4 6	14 7	7 4	7 1	22 1	14 6	2 9	16 0	16 9
Leucines		13 0	10 3	7 7	13 1	10 1	6 5	18 6	23 1
Phenylalanine		15 5		8 3	9 2	8 8	8 9	27 7	
γ-Aminobutyric	21 0	189 0	21 2	120 4	56 8	183 4	16 2	222 0	163 0
Total mcg /Gm	131 9	396 4	215 9	433 9	777 1	1,119 9	128 6	3,771 0	6,798 0

<sup>a</sup> Amino acid mcg /Gm fresh weight

amino acid such as proline should have constituted over 40% of the soluble nitrogen at all levels

The total soluble nitrogen produced was greater at each level of ammonia nitrate than with nitrate-nitrogen alone. The major portion of the increase was due to the amides, asparagine, and glutamine at the higher nitrogen levels. The appearance of asparagine at the higher levels of ammonia nitrate was accounted for by a reduction in the amount of aspartic acid in this series when compared with that supplied with nitrate. The combination of glutamic acid and glutamine under ammonia nutrition was more than three times that under the comparable nitrate nutrition, whereas the combination of aspartic acid and asparagine was roughly the same under both treatments.

The total amount of soluble nitrogen present in the stem (Table V) was approximately three times that found in the leaf. Aspartic and glutamic acids increased in the same proportions throughout the treatments, i.e., the aspartic acid was about three times the glutamic acid in most cases. Their amides also increased throughout the treatments but by contrast, glutamine was about three times the asparagine in all cases.

Phenylalanine, tyrosine,  $\beta$  alanine were not detected in leaf tissue but were present in quantitative amounts in the stem.

The total amount of soluble nitrogen present in the roots (Table VI) that had been furnished nitrate was only half that present in the leaf tissue, but roots furnished the high level of  $\text{NH}_3\text{NO}_3$  contained more than twice the soluble nitrogen of their leaves. This large amount of soluble nitrogen was predominantly asparagine and glutamine, indicating that the ammonia source stimulated the accumulation of amide in root tissue.  $\gamma$ -Aminobutyric acid is a major fraction in root tissue, particularly at the high levels of both  $\text{NO}_3$  and  $\text{NH}_3\text{NO}_3$ . It was observed that an abundance of  $\gamma$  aminobutyric acid seemed to be associated with

the presence of large amounts of alkaloid in the root.

**Alcohol-Insoluble Nitrogen (Protein)**—Although the nutrient supply had a pronounced effect on the metabolism of all plant parts, relatively slight changes in the amino acid composition of their protein was observed (Compare Tables IV and VII, V and VIII, VI and IX). All of the amino acids present in the soluble fraction except  $\gamma$ -aminobutyric acid occurred much more abundantly in the protein than in the free state. The ratio of aspartic acid to glutamic acid in the leaf protein varied from 2:1 to 3:1 with the greater ratio being associated with the higher nitrogen levels. Serine, glycine, threonine, alanine, valine, phenylalanine, and tyrosine remained substantially constant throughout all of the treatments. All levels of ammonia nitrate produced more protein than the corresponding nitrate treatment. Lysine and arginine commonly found in the protein fraction of plants were not detected in either the alcohol-soluble or insoluble fractions of the belladonna plant.

The composition of the stem protein (Table VIII) was similar to that of the leaf protein, but amounted to only one-tenth that of the leaf except at lowest  $\text{NO}_3$  levels. The ratio of aspartic acid to glutamic acid in the  $\text{NO}_3$  series was 2:1, in the  $\text{NH}_3\text{NO}_3$  it was 1:1, indicating that  $\text{NH}_3$  tends to stimulate the production of glutamic acid over aspartic acid.

The protein synthesized in the root (Table IX) contained the compounds previously mentioned in leaf and stem and contained, in addition, cysteine acid in all treatments. It is surprising that this is the only sulfur containing amino acid found in the protein fraction. The amount of protein synthesized in roots was about one-fourth that present in leaf and slightly more than twice that in stem. The compounds most noticeable in the root protein were the leucines, tyrosine, proline, and hydroxyproline. Of these, hydroxyproline is present in greater quantity than in any other plant part.

TABLE VII—COMPOSITION OF THE ALCOHOL INSOLUBLE NITROGEN FRACTIONS OF *Atropa belladonna* LEAF AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solution mg Equiv / liter						$\text{NO}_3\text{NH}_3$		
	5	10	15	20	25	30	5	20	30
Cysteic from cysteine									
Aspartic	2,140	3,000	5,652	2,601	5,690	6,584	5,242	3,536	3,905
Glutamic	970	1,000	2,314	1,910	1,993	1,960	2,065	1,852	2,009
Serine	185	800	1,291	296	998	834	974	1,310	1,988
Glycine	430	700	1,045	679	882	919	550	596	1,865
Asparagine									
Threonine	910	1,100	1,394	1,240	1,159	1,100	1,099	1,352	1,606
Alanine	648	1,050	1,842	2,125	2,170	1,439	1,077	2,909	2,046
Glutamine									
Proline	730	1,200	2,883	1,425	3,000	3,952	1,319	4,660	4,470
Valine	1,175	1,425	1,833	1,668	1,880	2,100	1,391	2,490	2,625
Leucines	2,310	2,700	3,766	3,070	4,067	4,645	3,312	6,577	5,551
Phenylalanine	1,010	1,220	1,428	1,363	1,388	1,440		1,979	2,139
Tyrosine	747	570	553	503	631	744	690	930	695
$\beta$ Alanine									
$\gamma$ Aminobutyric									
OH Proline			266	175	247	184	276	552	423
Total mcg /Gm	11,355	14,765	24,267	17,055	24,105	25,900	17,995	28,743	29,322

<sup>a</sup> Amino acid mcg /Gm fresh weight

TABLE VIII — COMPOSITION OF THE ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF *Atropa belladonna* STEM AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solution, mg Equiv /Liter						NO <sub>3</sub> NH <sub>4</sub>		
	5	10	15	20	25	30	5	20	30
Cysteine from cysteine									
Aspartic	450	476	508	656	417	531	525	593	519
Glutamic	168	257	296	390	244	245	240	462	429
Serine		112	103	125	58	103	76	132	131
Glycine	50	104	129	145	93	149	66	129	120
Asparagine									
Threonine	110	100	142	187	77	124	47	53	50
Alanine	135	176	191	221	187	145	161	158	183
Glutamine									
Proline	25	303	249	541	294	37	20	325	625
Valine	247	240	229	282	237	238	185	250	245
Leucines	285	398	225	512	431	413	380	431	425
Phenylalanine	150	175	188	250	193	112	165	181	263
Tyrosine			78	120	119	95	82	97	104
β-Alanine									
γ-Aminobutyric									
OH Proline		140	139	171	75	75		165	153
Total mcg /Gm	1,630	2,481	2,478	3,600	2,425	2,266	1,947	2,976	3,247

<sup>a</sup> Amino acid, mcg /Gm fresh weightTABLE IX — COMPOSITION OF THE ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF *Atropa belladonna* ROOT AS AFFECTED BY VARIOUS CONCENTRATIONS OF NITROGEN AFTER GROWTH IN WATER CULTURE FOR FIFTY-FIVE DAYS<sup>a</sup>

Amino Acids	Nitrogen in Nutrient Solution mg Equiv /Liter						NO <sub>3</sub> NH <sub>4</sub>		
	5	10	15	20	25	30	5	20	30
Cysteine from cysteine	220	270	300	260	262	260	220	240	250
Aspartic	594	682	1,456	543	560	403	449	608	722
Glutamic	226	232	650	672	622	662	273	253	453
Serine	106	123	594	336	269	248	130	350	341
Glycine	177	255	360	299	269	208	163	575	351
Asparagine									
Threonine	220	204	636	368	345	338	332	525	360
Alanine	270	255	385	391	356	351	291	490	361
Glutamine									
Proline	428	272	1,540	634	1,189	1,182	695	1,291	2,070
Valine	321	528	666	585	529	522	528	717	667
Leucines	490	773	1,143	1,026	899	880	843	1,322	1,176
Phenylalanine	285	403	428	381	426	429	317	469	426
Tyrosine	130	310	316	286	242	278	225	300	317
β-Alanine									
γ-Aminobutyric									
OH Proline	376	570	459	550	522	663	429	653	645
Total mcg /Gm	3,843	4,877	9,943	6,331	6,490	6,404	4,895	7,793	8,139

<sup>a</sup> Amino acid, mcg /Gm fresh weight

Alkaloids.—Regardless of the amount of nitrate furnished, the amount of alkaloid found in the leaf was approximately 1.5 mg /Gm dry weight (Tables X and XI). It indicates that inorganic nitrogen supplied in minimal amounts or more has little effect on the per cent of alkaloid in the leaf. There was a regular progression of absolute amounts of alkaloid in the leaf tissue with increasing nitrate concentration. Since the rate of alkaloid synthesis was fairly constant, this progression is taken to be largely an indication of the rate of tissue production.

The relative amount of alkaloid contained in stem tissue was roughly three-fourths that present in the leaf. The notable exception was 30 mg equiv where alkaloid content was as high as leaf alkaloid. The same relationship applied to alkaloid synthesis under ammonia nutrition.

The notable feature of alkaloid synthesis was its

high relative concentration in root tissue. This was unusually striking at 5 mg equiv of nitrate where 3.0 mg of alkaloid were present per Gm of dry tissue. This indicates that nitrogen was used in alkaloid synthesis in preference to that available for protein synthesis and growth. The absolute amounts of alkaloid present in root closely approximated that of leaf and stem combined. This was obviously due to the larger amounts of dry weight in leaf and stem.

Alkaloid synthesis under NH<sub>3</sub> nutrition exceeded that of plants furnished NO<sub>3</sub> at the higher nitrogen levels of both relative and absolute bases. These facts indicate that alkaloid synthesis has taken place in the root and the product was then translocated to overground plant parts. It is important to note that where the larger amounts of alkaloid were present in root, greater amounts of γ-aminobutyric

TABLE X—TOTAL ALKALOIDS OF BELLADONNA/GM OF DRY TISSUE<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	1 61	1 73	1 45	1 58	1 67	1 66	1 86	1 53	1 57
Stem	1 06	0 91	1 10	1 05	1 14	1 48	1 34	1 16	1 23
Root	3 04	1 76	2 52	2 90	3 09	2 50	2 86	4 02	3 90
Flowers	1 16	1 26	1 50	1 60	1 64	1 97	1 30	1 46	1 68
Total plant, av. mg /Gm	1 72	1 41	1 64	1 78	1 88	1 90	1 84	2 04	2 09

<sup>a</sup> Figures are mg of total alkaloidsTABLE XI—ABSOLUTE AMOUNTS OF TOTAL ALKALOIDS<sup>a</sup>

	Nitrate						Ammonia Nitrate		
	5	10	15	20	25	30	5	20	30
Leaf	5 54	7 27	9 18	11 57	10 05	10 72	5 65	11 38	12 14
Stem	6 19	7 67	9 27	9 69	9 25	11 78	6 86	12 13	14 02
Root	9 42	5 54	9 00	11 36	10 41	9 13	10 55	16 00	16 65
Flowers	1 02	1 64	2 10	2 53	2 16	2 42	1 30	2 66	4 03
Total plant	22 80	24 08	29 55	35 15	31 87	34 05	24 36	42 17	46 84

<sup>a</sup> Figures are mg of total alkaloids per plant partTABLE XII—TOTAL NITROGEN IN THE NITROGENOUS FRACTIONS OF THE LEAF, STEM, AND ROOT OF *Atropa belladonna* L.<sup>a</sup>

Solution Compn., mg Equiv N/L	NO <sub>3</sub>						NH <sub>3</sub> NO <sub>3</sub>		
	5	10	15	20	25	30	5	20	30
Nitrate (nitrogen)	2 50	4 77	11 06	19 40	26 29	32 95	2 52	11 15	9 61
Nitrite (nitrogen)									4 69
Ammonia (nitrogen)		2 71	3 36	7 07	10 23	11 41		11 84	15 39
Amide (nitrogen)	1 74	23 89	41 25	40 32	42 67	41 93	2 61	100 85	175 29
Soluble amino acid (nitrogen)	11 05	36 99	52 22	54 82	64 79	65 91	10 23	130 60	208 32
Protein (nitrogen)	62 96	11 85	216 99	177 61	235 25	269 24	86 81	277 35	355 20
Alkaloid (nitrogen)	0 87	0 99	1 30	1 56	1 40	1 51	1 11	1 90	2 04
Total mg of N fractions	77 38	157 31	284 93	260 46	337 96	381 02	100 67	432 84	595 25
Total N by Kjeldahl	173 00	279 00	373 70	451 70	528 00	547 00	161 00	515 00	665 00
Total nitrogen furnished per plant	192 00	385 00	578 50	770 00	962 00	1,130 00	192 00	770 00	1,130 00
Total nitrogen remaining in solution after growth period	0 00	0 00	150 00	250 51	312 50	525 00	0 00	180 00	400 00

<sup>a</sup> Numbers are total mg of nitrogen as the fraction listed per plant, mean of 12 plants.

acid and smaller amounts of proline were present in the soluble nitrogen fractions than that found in the other plant parts

**Interrelation of the Nitrogen Fractions.**—The nitrogenous compounds that tend to increase with increasing nitrate source are nitrate, ammonia, and free amino acids (Table XII). Those that tend to decrease are amides, protein, and alkaloids. With NH<sub>3</sub>-NO<sub>3</sub> supplied plants, nitrate decreased but there was a marked increase of free amino acids. The percentage of protein and greatly increased percentage of free amino acids indicates that at a high ammonia supply the amino acids were not rapidly combined into protein and therefore accumulated as free compounds.

## DISCUSSION

At the close of the growth experiments Kjeldahl determinations were made on the nutrient solutions to determine the amount of nitrogen present (Table XII). At the lower levels all of the nitrogen had been taken up by the plant. From 15 mg equiv of nitrogen, progressively larger amounts were still present and available. The summation of the

various fractions did not equal the total nitrogen determined by the Kjeldahl method because the fractions determined were not exhaustive and, to an extent, overlapped. Because of the small amount of tissue available the nitrogenous fractions of flowers were not determined.

Ammonia taken in by the roots was metabolized directly but at the higher levels was also accumulated. This was almost entirely in the stem. The only places where NO<sub>2</sub> was detected in tissue was in the root, where small amounts were present, and in the stems where larger amounts were observed. These were produced in plants furnished the highest NH<sub>3</sub>-NO<sub>3</sub> level. The larger amount of NH<sub>3</sub> already present in tissue probably hindered the conversion of NO<sub>2</sub> to NH<sub>3</sub>, causing it to accumulate under these conditions.

The major amino acids occurring in most plant parts included aspartic acid and its amide, asparagine, glutamic acid and its amide, glutamine, and  $\gamma$ -aminobutyric acid, which is thought to be formed by decarboxylation from glutamic acid. Variation among these compounds accounted for the majority of the changes in the soluble nitrogen fraction.



In leaf and root all were present except asparagine on nitrate nutrition, whereas in stem, asparagine was present at all levels of both sources of nitrogen. Levitt (8) has indicated that while glutamine is generally associated with processes involving synthesis, asparagine appears to be related to hydrolysis of protein and storage of soluble nitrogen. Asparagine, which was present on  $\text{NH}_4\text{NO}_3$  nutrition, has been described as a method of rendering high tissue concentration of  $\text{NH}_4$  innocuous.

Proline was present generally in large amounts in leaf and stem tissue under all nitrogen levels, and accumulated in roots where the alkaloid concentration declined. It appears reasonable that proline could be linked with an oxidized dicarboxylic acid fraction to produce the tropane nucleus. The fact that free proline was present in roots only at the high nitrogen concentration and where alkaloid synthesis was low, or curtailed, would indicate that the proline was used in synthesis at lower nitrogen concentrations, and some unexplained cause was responsible for the blocking of this synthesis at higher nitrogen levels with a consequent accumulation of proline. Hydroxyproline was present in the root protein in quantity where proline concentration increased. The reactions by which proline could be used in the synthesis of lysoeyamine might also be the same for the conversion of hydroxyproline into scopolamine, which is also present. From the data it would appear that the  $\gamma$ -aminobutyric acid might serve as one possible precursor of the alkaloid since its concentration decreased as alkaloid increased, and vice versa. The function of  $\gamma$  aminobutyric acid is probably a raw material for carboxylation and oxidation reactions in the production of a tropane nucleus. James (9) indicated that the  $\gamma$  type amino nitrogen compound is effective in alkaloid synthesis.

The composition of the plant proteins was much less subject to variation with nutritional treatment than any of the nitrogenous fractions. Since protein is a product of metabolic processes rather than an intermediate, it is to be expected that its degree of stability would be much greater. The largest fraction of leaf and stem protein was aspartic acid, which reached a ratio of 3:1 over glutamic acid at high nitrogen levels, indicating that the belladonna plant prefers the four carbon dicarboxylic acids in its metabolism in preference to the five carbon acids stemming from glutamic acid. At all levels of  $\text{NH}_4$  the plant produced more protein than comparable plants furnished nitrate. The advantage gained by furnishing the plant a reduced form of nitrogen appears to have its effect up to and including the synthesis of protein. The small amount of stem protein (one-tenth that of leaf) is explained in the fact that the majority of stem is composed of vascular and structural elements. It is reasonable that the high per cent of soluble nitrogen found in stem was present in these conducting elements—products of leaf and root synthesis in the process of translocation. The protein of the root was similar in composition to that of the leaf but in smaller amount.

The synthesis of alkaloid does not appear directly related to the concentration of simple nitrogenous sources. The assumption that alkaloid is synthesized in root was verified by its high concentration in root at all nitrogen levels. It is surprising

that in the translocation to overground parts, the relative amount present in leaf is greater than that in stem. This may be partially explained by the fact that meristematic tissue tends to hold a high concentration of alkaloid. The alkaloid concentration in the flower buds was relatively high, further indicating absorption at points of high metabolic activity. The low concentration in stem is a result of fully differentiated conducting tissue and the small amount of matured storage tissue composing it.

Several workers, James (9), Cromwell (10), and Leete, Marion, and Spencer (11), have hypothesized the use of arginine and ornithine in the synthesis of belladonna alkaloids. The fact that neither of these compounds was detected chromatographically indicates that their importance in direct synthesis must be a minor one. The introduction of exogenous ornithine in excised leaf or root tissue with a consequent occurrence in alkaloid did not demonstrate the path of synthesis employed in the intact plant. Elucidation of the actual pathway of synthesis must be demonstrated with a complete system that is not subject to drastic external constraints on normal equilibrium.

Certainly demonstration of the roles that proline, hydroxyproline, and  $\gamma$  aminobutyric acid play in the synthesis of the belladonna alkaloids is well worth further study along this line.

## SUMMARY

Belladonna plants grown on six levels of nitrate and three levels of  $\text{NH}_4\text{NO}_3$  were harvested and the following determinations were made on each plant part: nitrate, nitrite, ammonia, amides, free amino acids, protein (following hydrolysis), alkaloids, and total nitrogen.

1. There was a regular progression of  $\text{NO}_3$  concentration in the tissue with increasing  $\text{NO}_3$  supplied, extending to the point of accumulation far beyond the tissue requirements. The same thing applied to ammonia in a lesser degree only at high levels of  $\text{NH}_4\text{NO}_3$  supply.

2. Nitrite was detected in tissue only at highest levels of  $\text{NH}_4\text{NO}_3$  indicating its transient condition in nitrogen synthesis.

3. The concentration of  $\text{NH}_4$  in tissue was much less than that of  $\text{NO}_3$  and was confined in quantity to the stem.

4. The amides were present at high levels of  $\text{NO}_3$  and  $\text{NH}_4\text{NO}_3$  and amounted in the stem to four times the quantity in leaf and/or root.

5. The free amino acids amounted to one-tenth or more of the total nitrogen present. The following compounds were shown to be present at least in one plant part: cystic acid, aspartic acid, glutamic acid, serine, glycine, asparagine, threonine, alanine, glutamine, proline, valine, leucine, phenylalanine, tyrosine,  $\beta$  alanine,  $\gamma$  aminobutyric acid, and hydroxyproline.

The largest proportion of these compounds is composed of aspartic acid and its amide asparagine, glutamic acid and its amide glutamine,  $\gamma$ -aminobutyric acid, which is assumed to be made from glutamic acid by decarboxylation, and finally proline.

6. The  $\text{NH}_3\text{-NO}_3$  source stimulated greater formation of the 5-carbon dicarboxylic acids (glutamic and its relatives), while the  $\text{NO}_3$  source stimulated greater production of the 4-carbon acids (aspartic and its relatives).

7. The protein fraction was by far the largest, amounting to one-half or more of the total nitrogen. Its composition was fairly constant, being the result of metabolism rather than active metabolites. The protein of leaf and root was similar in composition and markedly different from stem, which served mainly as a storehouse of soluble nitrogen.

8. The alkaloid concentration was greatest in root on the  $\text{NH}_3\text{-NO}_3$  source, and coincided with small amounts of both free and combined proline. A possible mechanism whereby proline might be used in alkaloid synthesis is suggested. The relative concentration of alkaloid in leaf and flowers was substantially higher than that of stem, suggesting a number of problems involving its translocation.

9. Associated with the large amounts of alkaloid in roots are extremely large amounts of  $\gamma$ -aminobutyric acid, which could well fill the role of a  $\gamma$  semialdehyde as postulated by Vogel and Bonner (12) to be a precursor of alkaloid.

10. The rate of synthesis of nitrogenous fractions indicates protein and alkaloid are both products of a soluble nitrogen pool and are not directly interrelated in their synthesis or hydrolysis. The specific compounds mentioned; proline, hydroxyproline, and  $\gamma$ -aminobutyric acid, appear to be involved in the synthesis of alkaloid more than the majority of the free amino acids which are largely involved in protein synthesis.

## REFERENCES

- (1) Schermeister, L. J., Crane, F. A., and Voigt, R. F., *THIS JOURNAL*, 49, 694 (1960)
- (2) Varner, J. E., Bulen, W. A., Vanecko, S., and Burrell, R. C., *Anal. Chem.*, 25, 1528 (1953)
- (3) Thompson, J. F., and Steward, F. C., *Plant Physiol.*, 26, 375, 421 (1951)
- (4) Brown, N., Kirch, E. R., and Webster, G. L., *THIS JOURNAL*, 37, 24 (1948)
- (5) "U. S. Pharmacopeia XIV," Mack Printing Co., Easton, Pa., 1950
- (6) Ma, T. S., and Zuazaga, G., *Ind. Eng. Chem.*, 14, 280 (1942)
- (7) Steward, F. C., and Preston, G., *Plant Physiol.*, 15, 23 (1940)
- (8) Levitt, J., "Plant Physiology," Prentice Hall, Inc., New York, N. Y., 1954
- (9) James, W. O., *Nature*, 159, 196 (1947)
- (10) Cromwell, B. T., *Biochem. J.*, 37, 717 (1943)
- (11) Leete, E., Marion, L., and Spenser, I. D., *Nature*, 174, 650 (1954)
- (12) Vogel, H. J., and Bonner, D. M., *Proc. Natl. Acad. Sci. U. S. A.*, 40, 688 (1954)

# Preparation of 1-Alkyl-2,5-piperazinediones\*

By WILLIAM O. FOYE and DOUGLAS H. KAY†

General methods for the preparation of 1-alkyl-2,5-piperazinediones were found in the cyclization of N-alkylglycylglycines and the alkylation of the sodium salt of 1-acetyl-2,5-piperazinedione. 1-Methyl-2,5-piperazinedione showed significant antispasmodic activity.

LITTLE INFORMATION has been reported on the biological activity of 2,5-piperazinediones. Martinez (1) has reported that prior injection of 2,5-piperazinedione protected rats from diabetes caused by alloxan, and the growth of the embryo chick heart was reported to be favored by 2,5-piperazinedione (2). Fischer (3) has reported that 2,5-piperazinedione showed no antibacterial activity against *Staphylococcus*

*aureus*, but 3-methylene-2,5-piperazinedione showed good activity vs this organism. Miura (4) found only a slight bacteriostatic effect against *Trichophyton gypsum* by 2,5-piperazinedione, but the 3,6-dimethyl derivative was synergistic with sulfanilamide against *Escherichia coli*. More recently, de Jongh (5) investigated a series of N,N'-substituted 2,5- and 2,6-piperazinediones and found marked central nervous system depressant action as well as some tranquilizing and antispasmodic activity in some of the compounds.

Since a number of oxygenated heterocycles have shown antispasmodic effects, it was con-

\* Received March 31, 1960, from the Department of Chemistry, Massachusetts College of Pharmacy, Boston.  
Abstracted from a thesis submitted by D. H. Kay in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1958.

Presented to the Scientific Section, A. P. H. A., Washington meeting, August 1960.

† White Laboratories, Inc. Fellow, 1955-1958.

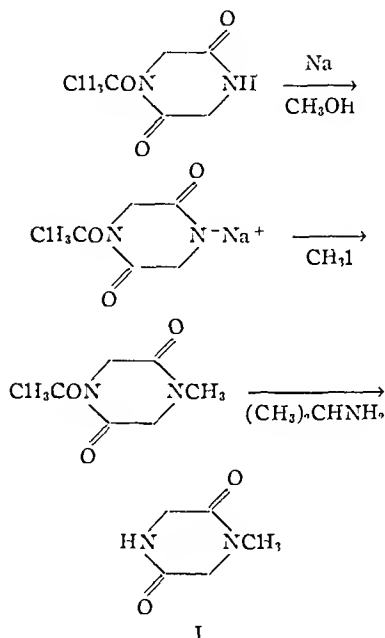
sidered that 2,5 piperazinedione derivatives might be worth while to examine for antispasmodic as well as other pharmacological effects. It was decided to prepare 1 alkyl 2,5 piperazinediones, since derivatives of this type in the piperazine series have shown a variety of pharmacological activities. With the exception of 1 methyl 2,5 piperazinedione, prepared by Chase and Downes (6) by cyclization of sarcosylglycine, no compounds of this type were found in the literature. A general method of preparation for 1 alkyl 2,5 piperazinediones was therefore investigated.

### METHOD

The use of several common acylating agents as possible blocking groups for one of the piperazinedione nitrogens was found unsuccessful in providing a reasonably stable monoacyl derivative of 2,5 piperazinedione. Treatment of 2,5 piperazinedione with ethyl chloroacetate, for instance, resulted in ring cleavage, and glycylglycine hydrochloride was obtained. Reactions with benzenesulfonyl chloride and ethanesulfonyl chloride also resulted in ring cleavage. Reaction with myristoyl chloride, however, gave both 1 myristoyl and 1,4 dimyristoyl 2,5 piperazinedione, which were easily separated. Treatment of 1 myristoyl 2,5 piperazinedione with methyl iodide did not give the desired 4 methyl derivative, but instead, 1,4 dimyristoyl 2,5 piperazinedione was produced. Reaction of the 1 myristoyl derivative with diethyl sulfate, however, gave 1,4 diethyl 2,5 piperazinedione, and reaction with benzenesulfonyl chloride likewise gave the 1,4 dibenzenesulfonyl derivative.

1 Acetyl 2,5 piperazinedione was obtained by a modification of Petrova's procedure (7) using an exchange reaction between the 1,4 diacetyl derivative and isopropylamine. The monoacetyl derivative was found to melt at 186–188°, but was shown by mixed melting point to differ from acetyl glycylglycine, reported by Fischer (8) to melt at 187–189°. Furthermore, preparation of the acetyldipeptide by Fischer's procedure (8) gave a product melting at 191–192°. Reaction of 1 acetyl 2,5 piperazinedione with acylating and alkylating agents generally resulted in ring opening, however. By first forming the sodium salt of 1 acetyl 2,5 piperazinedione in absolute methanol, alkylation was successful, and 1 acetyl 4 methyl 2,5 piperazinedione was obtained in good yield from reaction with methyl iodide. The acetyl group was readily removed by treatment with isopropylamine, and the resulting 1 methyl 2,5 piperazinedione was identical with that obtained by the procedure of Chase (6). This sequence of reactions, I, indicated that the method of Chase gave a cyclic product rather than an open chain peptide. Some doubt about the cyclization existed because of the ease of hydrolysis of 2,5 piperazinediones to peptides (9) as well as the similarity in melting points between 2,5 piperazinediones and their corresponding dipeptides in some cases.

A method resembling the cyclization procedure of Chase was found to be suitable for the preparation of 1 alkyl 2,5 piperazinediones and more convenient than the use of the sodium salt of 1 acetyl 2,5 piperazinedione. This method was based on the obser-



vation of Fisher and Fourneau (10) that ethyl glycylglycinate is readily cyclized in alcoholic ammonia to 2,5 piperazinedione. Accordingly, ethyl glycylglycinate was alkylated using methyl iodide, and the resulting N-methyl derivative was treated with dry ammonia to give the corresponding amide rather than the expected ring closure. Direct alkylation of glycylglycine was not attempted because of the insolubility of this compound in the common organic solvents. It was also necessary to neutralize the glycylglycine hydrochloride and convert it to anhydrous glycylglycine in order to isolate ethyl glycylglycine hydrochloride, which is soluble in the presence of water. In addition even traces of moisture prevent the crystallization of ethyl glycylglycine hydrochloride. Cyclization took place when the amide was refluxed in ethylene glycol. Several 1 alkyl 2,5 piperazinediones were prepared either by this method or that of Chase (6), illustrated by the preparation of 1 ethyl 2,5 piperazinedione, and their physical properties are listed in Table II. The corresponding properties of the intermediate N-alkylglycylglycines appear in Table I.

**Pharmacological Results**—Tests carried out by Dr. Howard J. Jenkins of the department of Biological Sciences on 1-methyl 2,5 piperazinedione showed this compound to have marked antispasmodic action on the rabbit ileum. Further pharmacological determinations will be reported.

### EXPERIMENTAL<sup>1</sup>

Melting points were taken on a Fisher Johns block and are uncorrected.

**1-Myristoyl- and 1,4-Dimyristoyl-2,5-piperazinedione**—To 90 cc of dimethylformamide was added 5.7 Gm (0.05 mole) of 2,5 piperazinedione, prepared by the procedure of Sannié (11), and the mixture was heated at 90° until most of the 2,5-piperazinedione was dissolved. Myristoyl chloride

<sup>1</sup> Analyses were done by Weiler and Strauss, Oxford, England, or by Carol K. Fitz, Needham, Mass.

TABLE I —N-ALKYLGLYCYLGLYCINES:  $\text{RNHCH}_2\text{CONHCH}_2\text{CO}_2\text{H}$ 

R	M P °C	Purification Solvent	Yield %	Formula	Analyses			
					Carbon Calcd	% Found	Hydrogen Calcd	% Found
$\text{C}_2\text{H}_5$	200–202	95% Ethanol	79	$\text{C}_6\text{H}_{12}\text{N}_2\text{O}_3 \cdot \text{H}_2\text{O}$	40 44	40 88	7 92	8 13
$\text{t-C}_4\text{H}_7$	213–215	95% Ethanol	72	$\text{C}_7\text{H}_{14}\text{N}_2\text{O}_3$	48 26	48 53	8 10	7 82
cyclo- $\text{C}_6\text{H}_{11}$	211–213	95% Ethanol	27	$\text{C}_{10}\text{H}_{18}\text{N}_2\text{O}_3$	56 05	55.84	8 46	8 41
$\text{C}_6\text{H}_5\text{CH}_2$	208–210	Abs ethanol	21	$\text{C}_{11}\text{H}_{14}\text{N}_2\text{O}_3$	59 44	60 02	6 34	5 97

TABLE II —1-ALKYL-2,5-PIPERAZINEDIONES:  $\text{R}-\text{N} \begin{array}{c} \text{O} \\ \parallel \\ \text{C} \\ \diagup \quad \diagdown \\ \text{N} \quad \text{NH} \\ \diagdown \quad \diagup \\ \text{C} \\ \parallel \\ \text{O} \end{array}$ 

R	M P °C	Purification Solvent	Yield, %	Formula	Analyses			
					Carbon Calcd	% Found	Hydrogen, Calcd	% Found
$\text{C}_2\text{H}_5$	172–173	Abs ethanol	48	$\text{C}_6\text{H}_{10}\text{N}_2\text{O}_2$	50 69	50 60	7 09	7 10
$\text{t-C}_4\text{H}_7$	198–200	95% Ethanol	38	$\text{C}_7\text{H}_{12}\text{N}_2\text{O}_2$	53 83	53 70	7 74	7 60
cyclo- $\text{C}_6\text{H}_{11}$	218–219	95% Ethanol	25	$\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_2$	61 21	60 70	8 22	8 10
$\text{C}_6\text{H}_5\text{CH}_2$	218–220	95% Ethanol	50	$\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_2$	64 72	64 70	5 93	6 00

(10.9 Gm, 0.05 mole, Eastman Organic Chemicals) was then added, and heating was continued for ten minutes with stirring. The hot reaction mixture was filtered and about 0.5 Gm of piperazinedione was recovered. The filtrate was placed in the refrigerator and after two hours was filtered. A yellow-green solid was obtained which was extracted with a total of 40 cc of hot benzene. About 0.5 Gm of 2,5-piperazinedione was obtained from the benzene-insoluble material. The hot benzene solution was decolorized with charcoal and concentrated, and 1.5 Gm (9%) of 1-myristoyl-2,5-piperazinedione was obtained after recrystallization from acetone, m p 145–146°.

*Anal*—Calcd for  $\text{C}_{18}\text{H}_{32}\text{N}_2\text{O}_4$ : C, 66.62, H, 9.94. Found: C, 66.56; H, 9.83.

The filtrate from the reaction mixture was evaporated to dryness *in vacuo*, and the resulting solid was extracted with hot acetone. The acetone solution was decolorized with charcoal and allowed to evaporate. Recrystallization of the resulting solid from ether gave 3.6 Gm (13%) of 1,4-dimyristoyl-2,5-piperazinedione which melted at 34–36°. This compound is hygroscopic.

*Anal*—Calcd for  $\text{C}_{32}\text{H}_{58}\text{N}_2\text{O}_4$ : C, 71.85, H, 10.93. Found: C, 72.02, H, 12.01.

*Reaction of 1-Myristoyl-2,5-piperazinedione with Methyl Iodide*—A mixture of 2.4 Gm (0.007 mole) of 1-myristoyl-2,5-piperazinedione, 75 cc of anhydrous benzene, and 1.1 Gm (0.007 mole) of methyl iodide was refluxed at 80° for four hours. The solution was cooled and 0.4 Gm of 2,5-piperazinedione was recovered. The remaining solution was evaporated *in vacuo* to a volume of about 5 cc, and 1.4 Gm of 1,4-dimyristoyl-2,5-piperazinedione was obtained, m p 36–38°.

*Reaction of 1-Myristoyl-2,5-piperazinedione with Diethyl Sulfate*—A mixture of 30 cc of benzene, 30 cc of 2% aqueous sodium hydroxide solution, 0.3 Gm (0.001 mole) of 1-myristoyl-2,5-piperazinedione, and 0.07 Gm (0.0005 mole) of diethyl sulfate was refluxed gently for two hours. A few drops of *n*-octanol prevented foaming. After cooling to room temperature the mixture was filtered. The resulting

solid was washed with hot water and hot benzene and then extracted with hot acetone to remove unreacted 1-myristoyl-2,5-piperazinedione. The remaining solid was recrystallized from 95% ethanol, and approximately 0.15 Gm of crystals were obtained which melted at 192–194°. Elemental analysis indicated the product to be 1,4-diethyl-2,5-piperazinedione, a reported m p for this compound is 129° (12).

*Anal*—Calcd for  $\text{C}_8\text{H}_{14}\text{N}_2\text{O}_2$ : C, 56.51, H, 8.32. Found: C, 56.50, H, 9.01.

*Reaction of 1-Myristoyl-2,5-piperazinedione with Benzenesulfonyl Chloride*—A mixture of 1.25 Gm (0.004 mole) of 1-myristoyl-2,5-piperazinedione, 0.68 Gm (0.004 mole) of benzenesulfonyl chloride, and 75 cc of water (with a few drops of sodium hydroxide solution to bring the pH to 8) was shaken for two hours. Additional alkali was added during this period to maintain a pH of 8. The mixture was then filtered, and unreacted 1-myristoyl-2,5-piperazinedione was recovered. The filtrate was evaporated to dryness *in vacuo*, and the resulting solid was extracted with hot absolute ethanol in a Soxhlet apparatus for twenty-four hours. The extract was concentrated to about 20 cc, and 0.2 Gm of white, crystalline material was isolated; m p 295–300° (with decomposition). Elemental analysis indicated it to be 1,4-dibenzoylenesulfonyl-2,5-piperazinedione.

*Anal*—Calcd for  $\text{C}_{16}\text{H}_{14}\text{N}_2\text{O}_6\text{S}_2$ : S, 16.24. Found: S, 15.98.

*Preparation of 1-Methyl-2,5-piperazinedione from 1-Acetyl-2,5-piperazinedione*—Fifty cubic centimeters of absolute methanol was treated with 0.6 Gm (0.026 mole) of sodium, and when solution was complete, 4 Gm (0.026 mole) of 1-acetyl-2,5-piperazinedione (7) was added. The reaction mixture was refluxed for one minute on a water bath, 11.5 Gm (0.08 mole) of methyl iodide was added, and the mixture was refluxed for five hours. The solvent was then removed *in vacuo* and a thick syrup was obtained which was taken up in 35 cc of anhydrous chloroform. Addition of 2 cc of isopropylamine gave a precipitate of sodium io-

dide which was filtered. Ether, 20 cc., was added to the filtrate, which was then allowed to stand at room temperature for three days with occasional shaking. The resulting solid was isolated, washed with warm ether, and recrystallized from 2-propanol. About 1.8 Gm (55%) of product was obtained which melted at 142–143°, which agrees with the reported value for 1-methyl-2,5-piperazinedione (6).

**Preparation of 1-Methyl-2,5-piperazinedione from Glycylglycine.**—*Glycylglycine*.—Twenty grams (0.11 mole) of glycylglycine hydrochloride monohydrate, prepared by the procedure of Fischer and Fournau (10), in 40 cc of water was treated with 10 Gm (0.11 mole) of sodium bicarbonate. The solution was evaporated to dryness *in vacuo*, and the residue was dissolved in a minimum amount of hot water. The solution was cooled and 50 cc of acetone was added. After the solution was stirred vigorously for several minutes, 20 cc of absolute ethanol was added, and a solid separated after fifteen minutes more of stirring. The product was filtered and washed with hot 95% ethanol. A yield of 13 Gm was obtained, the m p was 260–261°, with decomposition, which agrees with the reported value for glycylglycine (10).

*Ethyl Glycylglycinate Hydrochloride*.—Dry hydrogen chloride was bubbled into an ice-cooled mixture of glycylglycine (12.0 Gm, 0.09 mole) and 250 cc of absolute ethanol for one hour. The mixture was refluxed on a water bath for five minutes and filtered. After being cooled, the filtrate yielded 9.5 Gm of product which melted at 181–182°, which agrees with the reported value (10).

*Ethyl Glycylglycinate*.—The procedure of Fischer and Fournau (10) was used. From the previous product was obtained 4.5 Gm of ethyl glycylglycinate which melted at 89–90°, the reported value (10).

**1-Methyl-2,5-piperazinedione**.—A solution of 4.0 Gm (0.025 mole) of ethyl glycylglycinate, 30 cc of anhydrous chloroform, and 3.6 Gm (0.025 mole) of methyl iodide was refluxed for thirty minutes. The reaction mixture was filtered, and about 0.25 Gm of 2,5-piperazinedione was obtained. The filtrate was evaporated *in vacuo* to a thick syrup which was taken up in 40 cc of warm isopropyl alcohol. Dry ammonia was then passed into the ice-cooled mixture for one hour. The mixture was warmed on a water bath for ten minutes, cooled, and resaturated with dry ammonia. After standing at room temperature overnight, the mixture was filtered, and the residue was washed free of iodide ion with hot 95% ethanol. About 1.4 Gm of compound was obtained which decomposed at 280°.

This compound was refluxed in ethylene glycol for one hour, and ammonia was liberated. After removal of the solvent *in vacuo*, crystals were obtained on cooling the resulting syrup. The product was recrystallized from isopropyl alcohol and 0.6 Gm (19%) was obtained. The m p was 142–143°, the value reported for 1-methyl-2,5-piperazinedione (6).

**N-Ethylglycylglycine Monohydrate.**—A solution of 5.0 Gm (0.032 mole) of chloroacetyl glycine, prepared by the procedure of Levene, *et al* (13), and 30 cc of water was placed in an ice bath and treated slowly with 14 cc (0.22 mole) of 70% aqueous ethylamine solution with constant shaking. The solution was then allowed to stand for two days at room temperature, and was evaporated *in vacuo* to a thick syrup. The syrup was heated on a water bath with 100 cc of absolute ethanol, and crystallization began within fifteen minutes. After the mixture was cooled, 4.5 Gm of product was obtained which was washed with hot 95% ethanol, m p 200–202°.

*Anal*.—Calcd for  $C_6H_{12}N_2O_3 \cdot H_2O$ : C, 40.44, H, 7.92. Found: C, 40.88; H, 8.13.

**1-Ethyl-2,5-piperazinedione.**—A mixture of 4.0 Gm (0.022 mole) of N-ethylglycylglycine monohydrate and 30 cc of ethylene glycol was refluxed for thirty minutes. The mixture was evaporated *in vacuo* to a thick syrup which solidified on cooling. The residue was taken up in 75 cc of hot absolute ethanol and decolorized with charcoal. The solution was then concentrated to approximately half its original volume and cooled. A yield of 1.5 Gm of 1-ethyl-2,5-piperazinedione was obtained which melted at 172–173°.

*Anal*.—Calcd for  $C_6H_{10}N_2O_2$ : C, 50.69, H, 7.09. Found: C, 50.6; H, 7.1.

## REFERENCES

- (1) Martinez C, *Ret soc arg biol*, 26, 89(1930)
- (2) Odiette, D., and Truhaut, R., *Bull histol appl physiol et pathol et tech microscop*, 14, 97(1917)
- (3) Fischer, E., and Prizant, L., *Ret assoc med arg*, 69, 21(1935)
- (4) Miura, Y., Shibata, S., and Tozawa, T., *Igaku to Seibutsugaku*, 9, 13(1946), Shibata, S., and Miura, Y., *ibid.*, 10, 61(1947)
- (5) de Jongh, D. K., and van Proosdy-Hartzema, F. G., *J Pharm and Pharmacol*, 11, 393(1959)
- (6) Chase, B. H., and Downes, A. M., *J Chem Soc*, 1953, 3874
- (7) Petrova, R. G., Akimova, L. N., and Gavrilov, N. I., *Zhur Obshchei Khim*, 24, 2230(1954)
- (8) Fischer, E., and Otto, E., *Ber*, 36, 2115(1903)
- (9) Fischer, E., *ibid*, 38, 607(1905), Dunn, G., Newbold G. T., and Spring, F. S., *J Chem Soc*, 1949, S131
- (10) Fischer, E., and Fournau, E., *Ber*, 34, 2868(1901)
- (11) Sannic, C., *Bull soc chim*, 9, 487(1942)
- (12) Blek, L., Derkoseh, J., Michl, H., and Wessely, F., *Monatsh*, 84, 717(1953)
- (13) Levene, P. A., Sims, H. S., and Pfaltz, M. H., *J Biol Chem*, 61, 450(1924)

## The Permeability of Red Corpuscles to Various Sympathomimetic Amine Salts and Phenothiazine Derivatives

By EDWARD P. WINTERS† and WILLIAM J. HUSA

The effects of various sympathomimetic amine salts and phenothiazine derivatives in preventing hemolysis of human erythrocytes were studied. In some instances rabbit erythrocytes were used. The results indicated that in solutions of increasing concentrations of sympathomimetic amine salts, hemolysis initially decreased, then abruptly increased, followed by a second greater decrease and increase. In the presence of 0.6 per cent sodium chloride there was no hemolysis until a certain concentration of sympathomimetic amine salt was reached, which closely coincided with the concentration of the same drug that resulted in the final increase in hemolysis when sodium chloride was not present. Freezing point depressions and pH values of the compounds employed in the hemolytic method were determined. The compounds were tested for reactions with human serum and oxyhemoglobin. The phenothiazine derivatives tested were found to be unsuited for investigation by the hemolytic method.

PREVIOUS INVESTIGATORS (1-8) have emphasized the importance of utilizing the hemolytic method as a means of determining whether or not a solution calculated to be isotonic with the blood from physicochemical data is truly isotonic in the biological sense. It was noted that the osmotic pressure of a solute on the erythrocyte membrane depended not only on the number of solute particles in solution, but also upon whether or not the cell membrane was penetrated by these particles.

In the present investigation, different concentrations of various sympathomimetic amine salts and phenothiazine derivatives were tested in an attempt to evaluate their effect on red blood cells with respect to the prevention of hemolysis. The presence of 0.6 per cent sodium chloride gave indication of the effect of the sympathomimetic amine compounds upon erythrocytes when the sodium chloride concentration, in itself, was sufficient to prevent osmotic hemolysis.

## EXPERIMENTAL

**Collection of Blood.**—The method of obtaining blood in the present investigation was the same as previously described by Husa and co-workers (2, 3). The blood was drawn mainly from the veins of the arms of a thirty-two year old white male and occasionally from white volunteers.

**Preparation of Solutions.**—The solutions utilized in the present investigation were prepared in the same manner as previously described by other workers (2, 3).

\* Received December 17, 1959, from the College of Pharmacy, University of Florida, Gainesville.

This paper is based in part upon a dissertation presented by Edward P. Winters to the Graduate Council of the University of Florida, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Fellow of the American Foundation for Pharmaceutical Education, 1958-1959. Present address: Abbott Laboratories North Chicago, Ill.

**Quantitative Determination of Per Cent Hemolysis.**—Determinations of hemolytic *i* values were made in the same manner as previously described by Grosicki and Husa (2) and Easterly and Husa (3).

**Determination of pH Values.**—A Fisher titrimeter, No. 9-311A, equipped with calomel and glass electrodes, was utilized in all determinations of pH values.

**Determination of Precipitation on Mixing Compounds with Oxyhemoglobin.**—One per cent solutions of each compound were separately mixed with equal portions of oxyhemoglobin solutions prepared by laking human erythrocytes which had been previously washed in saline solution. The mixtures of the experimental compounds and oxyhemoglobin were allowed to stand in a water bath for forty-five minutes at 37°, centrifuged for three minutes at approximately 2,000 r. p. m., and observed for evidence of precipitation. Any precipitate would result in colorimetric readings lower than those which would ordinarily be obtained from the amount of oxyhemoglobin actually released from the erythrocytes.

**Determination of Precipitation on Mixing Compounds with Serum.**—Serum was separated from human erythrocytes by centrifugation as above and added in like portions to 1% solutions of each compound. The mixtures were observed for forty-five minutes, during which time they were in a water bath at 37°.

**Determination of Freezing Point Depression.**—The apparatus used to determine the freezing point depressions, unless otherwise indicated, was that of Bartley (9) as modified by Husa and Adams (1). The freezing point depressions of various concentrations of amphetamine sulfate solutions were determined since results with this compound exhibited great fluctuations in the degree of hemolysis.

**Chemicals.**—The chemicals employed in the present investigation were generously donated by their manufacturers: amphetamine sulfate, dextro-amphetamine sulfate, hydroxyamphetamine hydrobromide, chlorpromazine hydrochloride, prochlorperazine ethanedisulfonate, trifluoperazine dihydrochloride, and trimeprazine tartrate by Smith

Kline and French Laboratories, phenmetrazine hydrochloride by Geigy Pharmaceutical Co., methoxyphenamine hydrochloride by Upjohn Co., mephentermine sulfate by Wyeth Laboratories

RESULTS

**Results of Hemolytic Determinations**—The sympathomimetic amine salts employed did not give the usual sigmoid curve (see Fig 1) commonly obtained by this method with other compounds. Hemolysis in the presence of the sympathomimetic amine salts generally approached 100% in the lower concentrations, decreased as the drug concentration increased, then exhibited an abrupt increase in hemolysis again, only to be followed by a second decrease, which generally assumed the approximate proportions of a sigmoid curve (see Figs 2, 4, 5, 6, 7). This second decrease in hemolysis was usually followed by a sharp increase. Each hemolysis curve shown was obtained from results with one blood sample and is representative of other curves of the same compound in different blood samples.

Results with dextro amphetamine sulfate in both human and rabbit blood were very similar to those obtained using amphetamine sulfate in human blood.

Peculiar changes in hemolysis have been found with other compounds to a lesser degree by previous investigators (10, 11, 12) and hemolytic  $t$  values were calculated either by using only that part of the curve that was sigmoid and covered the greatest range of hemolysis, or by averaging the concentrations of the compound where the curve crossed the points of 25, 50, and 75% hemolysis more than once. In the present investigation, the former procedure was performed when possible. These hemolytic  $t$  values are compared in Table I with values of  $t$  obtained from the freezing point depressions of some of the compounds.

In the presence of 0.6% sodium chloride the con-

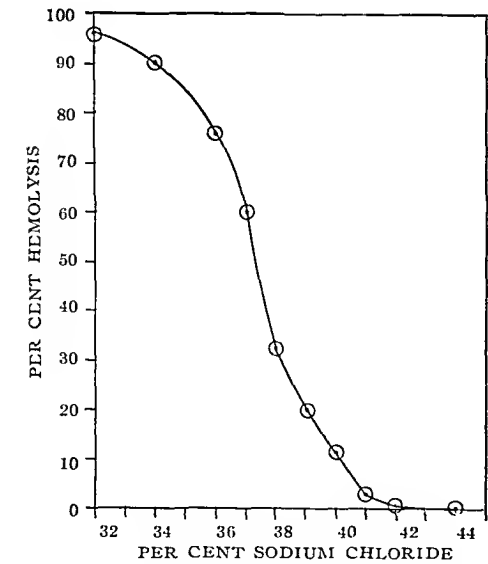


Fig 1—Typical curve resulting from hemolysis of human erythrocytes in sodium chloride solutions of various concentrations

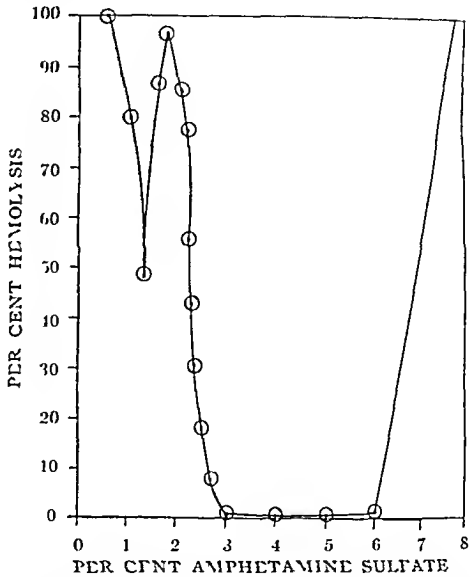


Fig 2—Hemolysis of human erythrocytes in amphetamine sulfate solutions of various concentrations

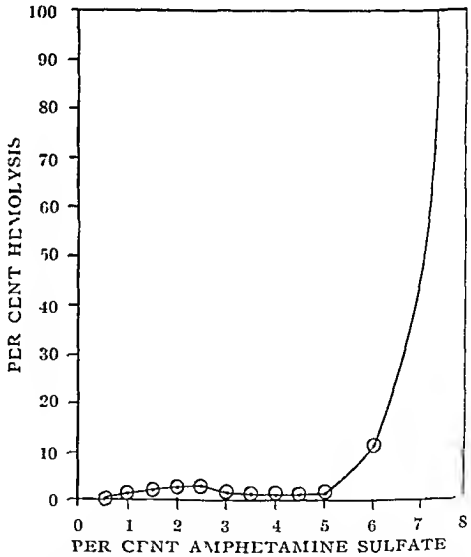


Fig 3—Hemolysis of human erythrocytes in amphetamine sulfate solutions of various concentrations in the presence of 0.6% sodium chloride

centrations of the dextro amphetamine sulfate and hydroxyamphetamine hydrobromide solutions employed exhibited no significant hemolysis. It is important to note that with the other compounds tested, no significant hemolysis occurred in the presence of 0.6% sodium chloride until a certain concentration was reached (see Figs 3, 5, 6, 7). Hemolysis then increased rapidly, frequently to readings on the colorimeter indicating more than 100% hemolysis, which would present the probability that some reaction had occurred between the compound and the blood components, resulting in a

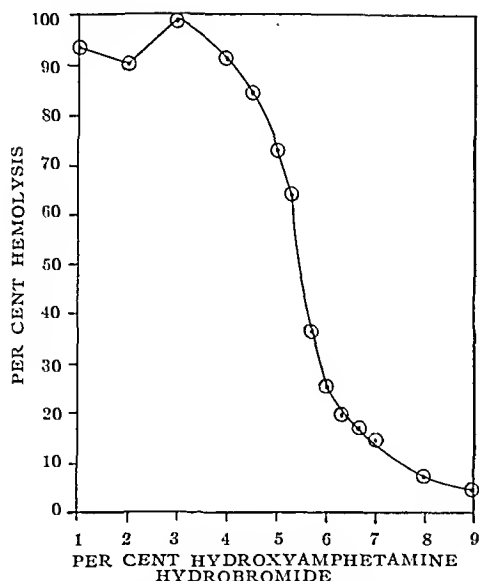


Fig. 4.—Hemolysis of human erythrocytes in hydroxyamphetamine hydrobromide solutions of various concentrations.

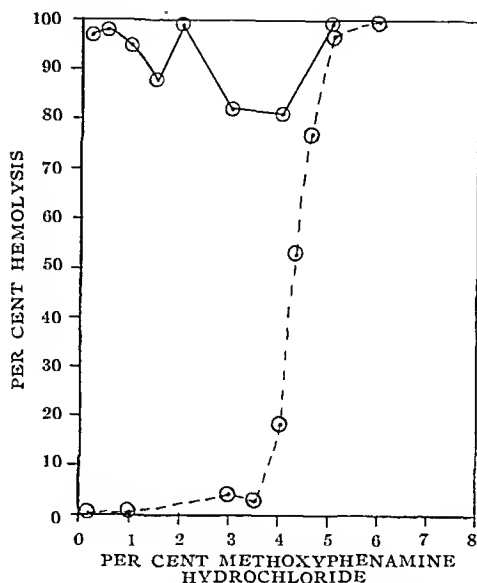


Fig. 6.—Hemolysis of human erythrocytes in various concentrations of methoxyphenamine hydrochloride in water (solid line) and in the presence of 0.6% sodium chloride solution (broken line).

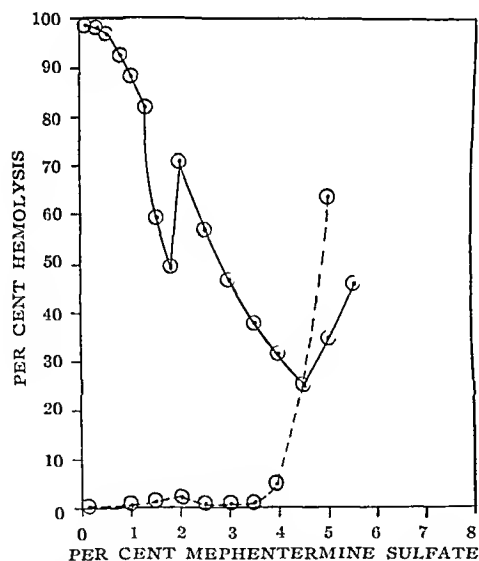


Fig. 5.—Hemolysis of human erythrocytes in various concentrations of mephentermine sulfate in water (solid line) and in the presence of 0.6% sodium chloride solution (broken line).

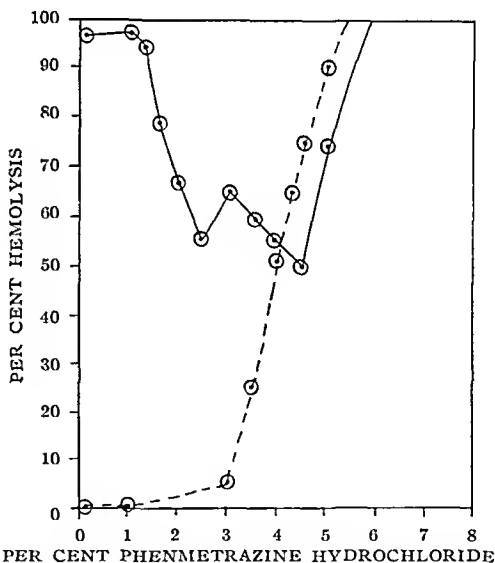


Fig. 7.—Hemolysis of human erythrocytes in various concentrations of phenmetrazine hydrochloride in water (solid line) and in the presence of 0.6% sodium chloride solution (broken line).

darkening of the mixture. This same concentration closely coincided with the final upward swing in the hemolysis curves of the same compounds without sodium chloride.

**Results of pH Determinations.**—For the compounds subjected to the hemolytic method, the concentrations chosen for pH determinations were those at which an abrupt change in the degree of hemolysis had occurred. Since amphetamine sulfate demonstrated quite wide variations in the percentage of hemolysis with small changes in drug

concentration (see Fig. 2), a more complete series of pH values was determined for this compound alone and in the presence of 0.6% sodium chloride. Phenothiazine derivatives were used in concentrations of 1% for the determination of the pH of these compounds. Some representative pH values obtained are listed in Table II.

The pH values of the sympathomimetic amine salt solutions employed ranged from 4.18 to 7.22 before the addition of human blood and 5.48 to 7.30 after



Kline and French Laboratories; phenmetrazine hydrochloride by Geigy Pharmaceutical Co; methoxyphenamine hydrochloride by Upjohn Co; mephentermine sulfate by Wyeth Laboratories

## RESULTS

**Results of Hemolytic Determinations.**—The sympathomimetic amine salts employed did not give the usual sigmoid curve (see Fig. 1) commonly obtained by this method with other compounds. Hemolysis in the presence of the sympathomimetic amine salts generally approached 100% in the lower concentrations, decreased as the drug concentration increased, then exhibited an abrupt increase in hemolysis again, only to be followed by a second decrease, which generally assumed the approximate proportions of a sigmoid curve (see Figs. 2, 4, 5, 6, 7). This second decrease in hemolysis was usually followed by a sharp increase. Each hemolysis curve shown was obtained from results with one blood sample and is representative of other curves of the same compound in different blood samples.

Results with dextro-amphetamine sulfate in both human and rabbit blood were very similar to those obtained using amphetamine sulfate in human blood.

Peculiar changes in hemolysis have been found with other compounds to a lesser degree by previous investigators (10, 11, 12) and hemolytic  $i$  values were calculated either by using only that part of the curve that was sigmoid and covered the greatest range of hemolysis, or by averaging the concentrations of the compound where the curve crossed the points of 25, 50, and 75% hemolysis more than once. In the present investigation, the former procedure was performed when possible. These hemolytic  $i$  values are compared in Table I with values of  $i$  obtained from the freezing point depressions of some of the compounds.

In the presence of 0.6% sodium chloride the con-

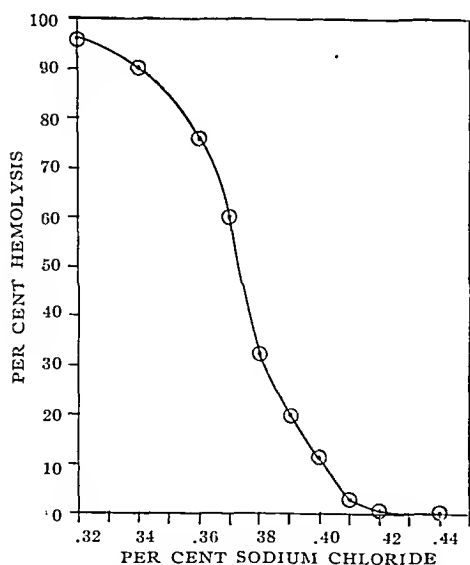


Fig. 1.—Typical curve resulting from hemolysis of human erythrocytes in sodium chloride solutions of various concentrations.

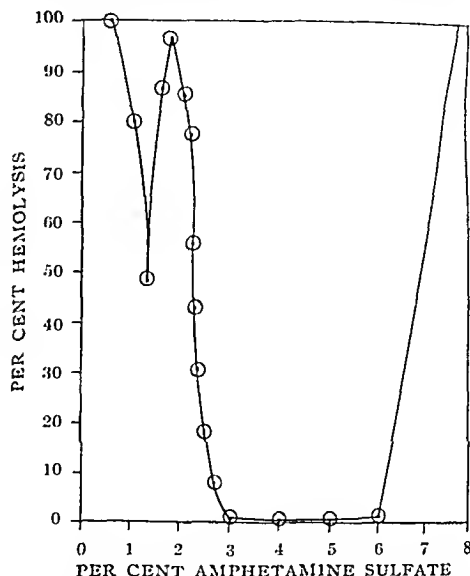


Fig. 2.—Hemolysis of human erythrocytes in amphetamine sulfate solutions of various concentrations

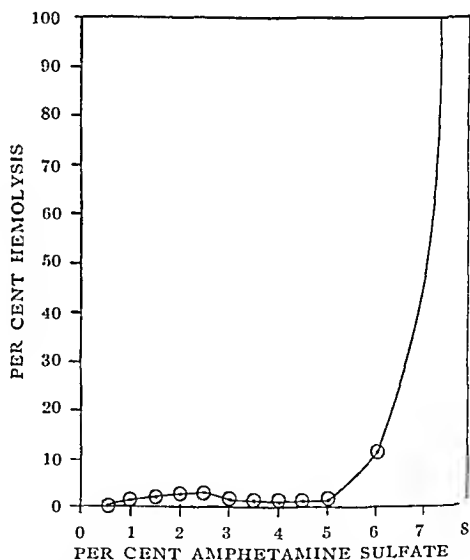


Fig. 3.—Hemolysis of human erythrocytes in amphetamine sulfate solutions of various concentrations in the presence of 0.6% sodium chloride.

centrations of the dextro-amphetamine sulfate and hydroxyamphetamine hydrobromide solutions employed exhibited no significant hemolysis. It is important to note that with the other compounds tested, no significant hemolysis occurred in the presence of 0.6% sodium chloride until a certain concentration was reached (see Figs. 3, 5, 6, 7). Hemolysis then increased rapidly, frequently to readings on the colorimeter indicating more than 100% hemolysis, which would present the probability that some reaction had occurred between the compound and the blood components, resulting in a

form of membrane destruction, other than that due to osmotic phenomena, would cause results different from those expected from purely colligative effects

It has been shown (15) that amine hydrochlorides may form micellar aggregates in solution with the result that their solutions have less osmotic effect than would be expected from the concentration used.

Variations in degrees of hemolysis can be partially explained not only on the basis of variations in colligative properties and membrane destruction, but also by variable reactions to the same compound by erythrocytes of the same blood sample. Love (16) and Hutchinson and Bean (17) investigated the per cent hemolysis-time curves for sodium alkyl sulfate and found that the curves were not the simple sigmoid type given by saponins, butanol, or streptolysin. The curves they obtained showed an initial rapid rate of hemolysis which decreased after a few minutes to zero, then slowly increased and continued in a sigmoid manner. Love (16) suggested that perhaps sodium dodecyl sulfate had both a protective and destructive effect. Protected cells evidently hemolyzed at a slower rate than normal cells.

Ponder (18) stated that cells of the same population would not necessarily react in a like manner to a given concentration of lysin. Differences in membrane composition or shape would result in different degrees of hemolysis at a given time from two samples of like concentrations of lysin.

It was further suggested by Ponder (19) that there may be two cell components involved in the resistance of an erythrocyte to hemolysis. The first component appears to be concerned with the mechanical rigidity and maintenance of the special shape of the cell and the other concerned with the prevention of the outward diffusion of pigment. The effects which the lysin produces on the first component are nearly always apparent before those which it produces on the second. It was theorized that some lysins affect the first component much more rapidly than they do the second, whereas others may produce their effects on the two components more nearly simultaneously.

## SUMMARY

1. Increasing concentrations of sympathomimetic amine salts resulted in rather unusual hemolysis curves.

2. The sympathomimetic amine salts did not cause hemolysis in the presence of 0.6 per cent sodium chloride until a certain concentration was reached which closely coincided with the concen-

tration of the salt alone, which resulted in the final sharp increase in hemolysis.

3. Both the *d* and *dl* forms of amphetamine sulfate gave results strongly indicative of a similar action on erythrocytes by the two compounds.

4. Determinations of the pH values before and after the addition of blood to various solutions of the sympathomimetic amine compounds indicated the acceptability of these compounds for testing by the hemolytic method.

5. No precipitation or color change occurred when solutions of laked erythrocytes and blood serum were mixed with 1 per cent concentrations of the sympathomimetic amine salts.

6. The phenothiazine derivatives employed could not be tested by the hemolytic method due to their reaction with blood.

7. The determination of the freezing point depression of various concentrations of amphetamine sulfate solutions negated the possibility of micelle formation of this compound in the concentrations used as being responsible for changes in its hemolysis curve.

## REFERENCES

- (1) Husa, W. J., and Adams, J. R., *THIS JOURNAL*, **33**, 329 (1944).
- (2) Grosicki, T. S., and Husa, W. J., *ibid.* **43**, 632 (1954).
- (3) Easterly, W. D., and Husa, W. J., *ibid.* **43**, 750 (1951).
- (4) Hartman, C. W., and Husa, W. J., *ibid.* **46**, 430 (1957).
- (5) Cadwallader, D. E., and Husa, W. J., *ibid.* **47**, 705 (1958).
- (6) Thomasson, L. C., and Husa, W. J., *ibid.* **47**, 711 (1958).
- (7) Ansel, H. C., and Husa, W. J., *ibid.* **48**, 516 (1959).
- (8) Zanolwjak, P., and Husa, W. J., *ibid.* **48**, 565 (1959).
- (9) Bartley, E. H., *Arch. Diagnosis*, **6**, 300 (1913).
- (10) Cadwallader, D. E., Jr., dissertation, "The Permeability of Red Corpuscles to Various Salts of Organic Acids," University of Florida, 1957.
- (11) Ansel, H. C., dissertation, "The Permeability of Red Corpuscles to Various Salts of Gluconic Acid," University of Florida, 1959.
- (12) Marcus, D., dissertation, "The Permeability of Red Corpuscles to Various Local Anesthetics," University of Florida, 1959.
- (13) West, E. S., and Todd, W. R., "Textbook of Biochemistry," The Macmillan Co., New York, N. Y., 1951, p. 602.
- (14) Pethica, B. A., and Anderson, P. J., *Koninkl. Vlaam. Acad. Wetenschap., Letter en Schone Kunsten Belg.*, **1953**, 129, through *Chem. Abstr.*, **49**, 2537 (1955).
- (15) Hammarlund, E. R., and Pedersen, B. Jergaard, K., *Dansk Tidsskr. Farm., Suppl.* **2**, 107 (1956).
- (16) Love, L., *J. Cellular Comp. Physiol.*, **44**, 291 (1950).
- (17) Hutchinson, E., and Bean, K. E., *Arch. Biochem. Biophys.*, **58**, 81 (1955).
- (18) Ponder, E., "Hemolysis and Related Phenomena," Grune and Stratton, New York, N. Y., 1948, p. 211.
- (19) *ibid.*, p. 31.

TABLE I—COMPARISON OF  $\iota$  VALUES OBTAINED FROM FREEZING POINT DEPRESSION DATA WITH THOSE OBTAINED FROM HEMOLYTIC RESULTS<sup>a</sup>

Compound	Species	$\iota$ Values—	
		Freezing Point <sup>b</sup>	Hemolytic <sup>c</sup>
Amphetamine sulfate	Human	2.40	1.97
Dextro amphetamine sulfate <sup>d</sup>	Human	2.40	2.03
Dextro amphetamine sulfate	Rabbit	2.40	1.57
Hydroxyamphetamine hydrobromide	Human	2.13	0.54
Hydroxyamphetamine hydrobromide	Rabbit	2.13	0.50

<sup>a</sup> Unless otherwise indicated, all hemolytic  $\iota$  values represent an average of two blood samples.

<sup>b</sup> Values of  $\iota$  determined from the freezing point depression of 1% solutions of the compounds (data obtained from manufacturer).

<sup>c</sup> Average of  $\iota$  values calculated at concentrations causing 20, 50, and 75% hemolysis.

<sup>d</sup> Average of three blood samples used to determine hemolytic  $\iota$  value.

the addition of human blood, thus being in the range in which pH has the least effect on hemolysis.

Of the phenothiazine derivatives used, prochlorperazine ethanedisulfonate and trifluoperazine dihydrochloride had pH values of less than 3.0 in concentrations of 1%. This, in itself, would exclude the use of these two compounds in the hemolytic method due to the fact that brown colored hematin is formed from oxyhemoglobin in the presence of solutions having low pH values (13).

**Results of Tests on Mixing Compounds with Oxyhemoglobin**—No precipitation was observed in any of the tubes containing 1% solutions of the compounds under investigation. Solutions of the phenothiazine derivatives turned brown upon admixture with laked erythrocytes.

**Results of Tests on Mixing Compounds with Serum**—Upon addition of human serum to solutions of the compounds under investigation, an immediate cloudiness was observed in every instance. This milk white cloud disappeared upon shaking in tubes containing sympathomimetic amine salts but remained in the tubes containing the phenothiazine derivatives.

Tests were also conducted in which defibrinated human blood was added to 1% solutions of the phenothiazine derivatives employed. When 0.1 ml of human blood was added to 10 ml of the solutions, the cells appeared to disperse into tiny red fragments. A gray precipitate soon appeared when the tubes were placed in a water bath at 37° and the solutions turned brown. The solution of trimetopazine tartrate contained more precipitate than the other phenothiazine derivative solutions. The precipitate which had formed in the trifluoperazine dihydrochloride solution disappeared upon agitation.

Due to the precipitates formed and the color changes of the solutions, the phenothiazine derivatives employed in this investigation could not be tested by the usual hemolytic method.

**Results of Freezing Point Depression Determinations**—The freezing point depressions of the amphetamine sulfate solutions tested failed to show cause for the unusual behavior of this compound when subjected to the hemolytic method.

TABLE II—pH VALUES FOR VARIOUS CONCENTRATIONS OF AQUEOUS EXPERIMENTAL COMPOUND SOLUTIONS BEFORE AND AFTER THE ADDITION OF HUMAN BLOOD<sup>a</sup>

Compound	Per Cent	Initial pH	pH 45 min After the Addition of Blood <sup>b</sup>
Blank (triple distilled water)		6.18	7.70
Amphetamine sulfate <sup>c</sup>	0.50	6.67	7.19
Amphetamine sulfate <sup>c</sup>	1.00	6.20	7.04
Amphetamine sulfate <sup>c</sup>	1.30	6.06	7.00
Amphetamine sulfate <sup>c</sup>	1.70	6.12	6.92
Amphetamine sulfate <sup>c</sup>	2.40	6.01	6.88
Dextro amphetamine sulfate <sup>c</sup>	0.50	6.38	7.30
Dextro amphetamine sulfate <sup>c</sup>	1.25	5.68	6.85
Hydroxyamphetamine hydrobromide <sup>c</sup>	2.00	5.63	6.67
Mephentermine sulfate <sup>c</sup>	1.80	4.59	6.99
Mephentermine sulfate <sup>c</sup>	2.00	4.50	6.95
Methoxyphenamine hydrochloride <sup>c</sup>	2.00	6.10	7.00
Methoxyphenamine hydrochloride <sup>c</sup>	3.00	6.05	6.99
Phenmetrazine hydrochloride <sup>c</sup>	1.00	4.82	5.95
Phenmetrazine hydrochloride <sup>c</sup>	2.00	4.60	5.80
Chlorpromazine hydrochloride <sup>d</sup>	1.00	5.60	5.65
Prochlorperazine ethane disulfonate <sup>d</sup>	1.00	2.70	2.80
Trifluoperazine dihydrochloride <sup>e</sup>	1.00	2.70	2.88
Trimetopazine tartrate <sup>f</sup>	1.00	5.70	6.00

<sup>a</sup> A 0.10 ml quantity of blood was added to 10 ml of solution.

<sup>b</sup> The pH of the blood initially was 7.92 and was 7.82 after forty-five minutes.

<sup>c</sup> Percentages chosen due to sharp change in degree of hemolysis at these concentrations.

<sup>d</sup> Solutions of these compounds turned brown upon addition of blood.

## DISCUSSION OF RESULTS

Factors influencing the degree of hemolysis of erythrocytes in various solutions are numerous. Hemolysis due to hypotonicity would be lessened by the simple expedient of adding more solute to the solution, thereby establishing a more nearly equal osmotic pressure between the solution and the cellular contents. A loss of electrolytes from within the cell into the external fluid would result in abnormally high hemolytic  $\iota$  values. The penetration into the erythrocyte by a divalent ion, such as the sulfate ion, would result in the release of two chloride ions from within the cell into the surrounding fluid in order to maintain electrical equilibrium, thus decreasing the number of particles within the cell. Slightly low  $\iota$  values may be indicative of incomplete dissociation, association, or micellar formation by the compound.

The hemolytic activity of many surface active lysins has been attributed to the collapse of a cholesterol phospholipid lipoprotein complex in the erythrocyte surface (14). Due to the apparent surface activity of the compounds employed in the present investigation, this could possibly be a factor in some of the hemolytic results obtained. Indeed, any

form of membrane destruction, other than that due to osmotic phenomena, would cause results different from those expected from purely colligative effects

It has been shown (15) that amine hydrochlorides may form micellar aggregates in solution with the result that their solutions have less osmotic effect than would be expected from the concentration used

Variations in degrees of hemolysis can be partially explained not only on the basis of variations in colligative properties and membrane destruction, but also by variable reactions to the same compound by erythrocytes of the same blood sample Love (16) and Hutchinson and Bean (17) investigated the per cent hemolysis-time curves for sodium alkyl sulfate and found that the curves were not the simple sigmoid type given by saponins, butanol, or streptolysin. The curves they obtained showed an initial rapid rate of hemolysis which decreased after a few minutes to zero, then slowly increased and continued in a sigmoid manner. Love (16) suggested that perhaps sodium dodecyl sulfate had both a protective and destructive effect. Protected cells evidently hemolyzed at a slower rate than normal cells.

Ponder (18) stated that cells of the same population would not necessarily react in a like manner to a given concentration of lysin. Differences in membrane composition or shape would result in different degrees of hemolysis at a given time from two samples of like concentrations of lysin.

It was further suggested by Ponder (19) that there may be two cell components involved in the resistance of an erythrocyte to hemolysis. The first component appears to be concerned with the mechanical rigidity and maintenance of the special shape of the cell and the other concerned with the prevention of the outward diffusion of pigment. The effects which the lysin produces on the first component are nearly always apparent before those which it produces on the second. It was theorized that some lysins affect the first component much more rapidly than they do the second, whereas others may produce their effects on the two components more nearly simultaneously.

### SUMMARY

1. Increasing concentrations of sympathomimetic amine salts resulted in rather unusual hemolysis curves.

2. The sympathomimetic amine salts did not cause hemolysis in the presence of 0.6 per cent sodium chloride until a certain concentration was reached which closely coincided with the concen-

tration of the salt alone, which resulted in the final sharp increase in hemolysis.

3. Both the *d* and *dl* forms of amphetamine sulfate gave results strongly indicative of a similar action on erythrocytes by the two compounds.

4. Determinations of the pH values before and after the addition of blood to various solutions of the sympathomimetic amine compounds indicated the acceptability of these compounds for testing by the hemolytic method.

5. No precipitation or color change occurred when solutions of laked erythrocytes and blood serum were mixed with 1 per cent concentrations of the sympathomimetic amine salts.

6. The phenothiazine derivatives employed could not be tested by the hemolytic method due to their reaction with blood.

7. The determination of the freezing point depression of various concentrations of amphetamine sulfate solutions negated the possibility of micelle formation of this compound in the concentrations used as being responsible for changes in its hemolysis curve.

### REFERENCES

- (1) Husa, W. J. and Adams, J. R., *This Journal*, **33**, 329 (1944).
- (2) Grosiecki, T. S., and Husa, W. J., *ibid.*, **43**, 632 (1954).
- (3) Easterly, W. D., and Husa, W. J., *ibid.*, **43**, 750 (1954).
- (4) Hartman, C. W., and Husa, W. J., *ibid.*, **46**, 430 (1957).
- (5) Cadwallader, D. E., and Husa, W. J., *ibid.*, **47**, 705 (1958).
- (6) Thomasson, L. C., and Husa, W. J., *ibid.*, **47**, 711 (1958).
- (7) Ansel, H. C., and Husa, W. J., *ibid.*, **48**, 516 (1959).
- (8) Zanowial, P., and Husa, W. J., *ibid.*, **48**, 565 (1959).
- (9) Bartley, E. H., *Arch. Diagnosis*, **6**, 300 (1913).
- (10) Cadwallader, D. E., Jr., dissertation, "The Permeability of Red Corpuscles to Various Salts of Organic Acids," University of Florida, 1937.
- (11) Ansel, H. C., dissertation, "The Permeability of Red Corpuscles to Various Salts of Gluconic Acid," University of Florida, 1959.
- (12) Marcus, D., dissertation, "The Permeability of Red Corpuscles to Various Local Anesthetics," University of Florida, 1959.
- (13) West, E. S., and Todd, W. R., "Textbook of Biochemistry," The Macmillan Co., New York, N. Y., 1951, p. 602.
- (14) Pethica, B. A., and Anderson, P. J., *Koninkl. Vlaam. Acad. Wetenschap., Letter en Schone Kunsten Belg.*, 1953, 129, through *Chem. Abstr.*, **49**, 2537 (1955).
- (15) Hammarlund, E. R., and Pedersen Rjergaard, K., *Dansk Tidsskr. Farm.*, Suppl. **2**, 107 (1956).
- (16) Love, L. J., *J. Cellular Comp. Physiol.*, **44**, 291 (1950).
- (17) Hutchinson, E., and Bean, K. E., *Arch. Biochem. Biophys.*, **58**, 81 (1955).
- (18) Ponder, E., "Hemolysis and Related Phenomena," Crane and Stratton, New York, N. Y., 1948, p. 211.
- (19) *ibid.*, p. 31.

# Canine Blood Sugar and Lactic Acid Responses to Adrenergic Amines after Ganglion Block\*

By R. S. McCUTCHEON

Since preliminary studies indicated that a ganglionic block before infusions of some adrenergic amines resulted in an increased production of blood lactic acid and blood sugar, it was postulated that this might be due to an interruption of Cori's cycle. This experiment shows that there is a significant increase in lactic acid produced by two of the drugs after blocking and an indication that two others also have this effect. The results on blood sugar were not significant. Evidence is presented here that the conversion of lactic acid to glycogen in the liver is blocked or inhibited by use of the ganglion blocking agent, mecamylamine, accounting for an accumulation of blood lactic acid under the conditions of the experiment.

SEVERAL investigators (1) have indicated that production of lactic acid is the mechanism by which smooth muscle is relaxed, and that the formation of lactic acid is the first function of epinephrine, not the release of glucose (2). Other investigators (3) have indicated that the increased lactic acid is the result of glucose metabolism (4, 5). Most of this work has been done on epinephrine alone and in only a few instances has the work been related to the effect of ganglion blocking agents (6, 7, 8). Preliminary work in our laboratory has indicated that the increase in blood lactic acid produced by injections of adrenergic amines is greater after a ganglionic block than without it. The potentiation of the lactic acid response under these conditions has led us to postulate that it may be due to a block or an inhibition of the synthesis of lactic acid to glycogen by the liver in Cori's cycle (9). If at the same time the production of lactic acid by muscle is increased by the amine there must be an accumulation of lactic acid. The present study was undertaken to further investigate this problem in relation to epinephrine and other adrenergic amines before and after ganglionic block, and as a preliminary to a similar study in relation to these effects after adrenergic block.

Thus, a comparative study was carried out in the anesthetized dog of the blood sugar and lactic acid effects after ganglionic block, using epinephrine, isoproterenol, ethylnorepinephrine, and

the N-isopropyl derivative of ethylnorepinephrine (Win-3046).

## EXPERIMENTAL

**Method.**—Mongrel dogs of either sex weighing 10 to 15 Kg. were anesthetized with pentobarbital sodium, 35 mg./Kg., intraperitoneally. No morphine nor other preanesthetic agent was used at any time. Blood sugar was determined according to the method of Nelson (10); lactic acid by the method of Barker and Summerson (11), after deproteinizing by the method of Folin-Wu, Van Slyke and Hawkins (12).

Constant infusions of the adrenergic drug were made into the jugular vein and samples were drawn from the saphenous vein of the hind leg. Control blood sugar and blood lactic acid were obtained before starting the infusion. Alternate dogs were given the ganglionic blocking agent, mecamylamine, 1 mg./Kg. and in some cases, 2 mg./Kg. When mecamylamine was used it preceded the infusion of the adrenergic agent. Control studies were also made in which mecamylamine was followed by infusions of physiological saline solution duplicating the infusions of the amines. No change in lactic acid production occurred. The amines were infused by means of a syringe actuated by a stepping relay controlled through an electronic counting device (13) or by an infusion pump manufactured by the Harvard Apparatus Co.

Infusions of increasing doses of the drugs lasting about two hours were used in order to determine degree of altered response to increasing doses.

TABLE I.—CONCENTRATION OF DRUGS AND RATE OF INFUSION\*

Time Intervals, min.	Epinephrine	Ethylnorepinephrine	Win-3046	Isoproterenol
1	0.153	2.15	2.54	0.111
2	0.613	4.3	5.07	0.444
3	1.033	8.6	10.14	1.14
4	1.533	10.74	12.68	1.7
5	3.066	21.40	25.30	2.28
6	4.599	42.80	50.75	4.56
7	9.200	86.00	101.48	9.12

\* Doses in mcg./Kg./min.

\* Received March 15, 1960, from the Department of Pharmacology, School of Pharmacy, Oregon State College, Corvallis.

The experimental work was instituted during tenure in Cardiovascular Research and Training Program, Medical College of Georgia, Augusta, supported in part by Grant H-TS-5044 from the National Heart Institute, Public Health Service. The work was completed at Oregon State College under grant H-3121, National Institutes of Health, Public Health Service.

The author wishes to express appreciation to Dr. R. P. Ahlquist for his counsel on this project, and to David A. McClure for his help with the chemical analysis.

Drugs were kindly furnished as follows: mecamylamine (Inversine) by Merck, Sharp and Dohme, and the amines used [ethylnorepinephrine (Butanephine), isoproterenol (Isuprel), and Win-3046] by Sterling-Winthrop Research Institute.

TABLE II.—BLOOD SUGAR AND BLOOD LACTIC ACID INCREASES BEFORE AND AFTER GANGLIONIC BLOCK

Drug	Average Increase without Block, mg. %				Average Increase after Block, mg. %				Differences, mg. %	
	Blood Sugar	No. Animals Used	Lactic Acid	No. Animals Used	Blood Sugar	No. Animals Used	Lactic Acid	No. Animals Used	Blood Sugar	Lactic Acid
Epinephrine	167	12	23	12	133	5	24	8	-35	+1
Ethylnorepi- nephrine	140	4	23	5	140	3	27	4	+0.4	+4
Win-3046	180	3	33	4	185	3	43	5	+5	+10
Isoproterenol	143	4	27	4	128	7	42	7	-16	+14

It was also of interest to determine whether or not the larger concentrations of drug would themselves have an inhibiting effect. Blood samples were analyzed at twenty-minute intervals during the infusion in order to determine what changes were taking place with increasing doses of the amine. Since in every case the increases in blood sugar and blood lactic acid reflected the increasing concentration of the infusion, the figures used in the reported results were the averages of the values obtained at the end of the infusion.

The solutions of the amines used contained 0.1% each of chlorobutanol and sodium bisulfite as preservative. The concentration of drug and rate of infusion are shown in Table I. Note that the dosage of the drugs has been adjusted at each level to give approximately equal effects in terms of known responses.

### RESULTS

The increases in blood sugar and blood lactic acid produced by the infusions before and after ganglion block were obtained by taking the differences between the control (value before injection of any drug) and the final value for each experiment. These results were averaged for each series of like experiments. Table II shows the figures, in mg. %, obtained in this way.

Statistically, this is a  $2 \times 4$  factorial experiment with disproportional numbers of observations in the subclasses. Without blocking and after blocking constitute one factor, and the four amines constitute another. The number of animals in the eight subclasses for lactic acid and blood sugar are shown in Table II.

TABLE III.—ANALYSIS OF VARIANCE FOR LACTIC ACID

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Drug	1,675.39	3	558.46	4.88 <sup>a</sup>
Blocking	563.32	1	563.32	4.92 <sup>b</sup>
Interaction	339.45	3	113.15	0.99
Error	4,689.98	41	114.39	..

<sup>a</sup> Significant at 1% level. <sup>b</sup> Significant at 5% level.

**Lactic Acid.**—The analysis of variance of lactic acid data is shown in Table III. The four sympathomimetic amines in the absence of a blocking agent produced statistically significant increases in the levels of lactic acid.

In the presence of the ganglion blocking agent, all four sympathomimetic amines led to statistically significant increases in lactic acid levels. In the case of two of the amines, Win-3046 and isoproterenol, the increase in lactic acid concentration was significantly greater with the blocking agent than without it. It can be said that the blocking agent had an effect on lactic acid levels and that in no case was the production of lactic acid blocked.

**Blood Sugar.**—The analysis of variance of blood sugar data is shown in Table IV. The four sympathomimetic amines in the absence of a blocking agent produced statistically significant increases in the levels of blood sugar.

In the presence of a blocking agent, all four sympathomimetic amines led to statistically significant increases in blood sugar levels. The difference between blocking and nonblocking and interaction are not significant. Examination of the data reveals that the experimental variation is large and the number of animals is small. Under these conditions, blocking is not significant.

### DISCUSSION

This study has shown that blood sugar and blood lactic acid are increased by infusions of the four amines: epinephrine, ethylnorepinephrine, Win-3046, and isoproterenol. This increase is large whether the infusion is made with or without the ganglion blocking agent mecamylamine. In the case of two of the amines, Win-3046 and isoproterenol, the increases of lactic acid were significantly greater after the use of the blocking agent.

An examination of the lactic acid differences of Table II is very suggestive since these appear in the same order as that postulated for the  $\beta$ -stimulating effects of these four substances (14, 15).

Since the results for blood sugar are not consistent and the differences in the increases before and after block are not significant, it would seem that the greater production of lactic acid after block is not

TABLE IV.—ANALYSIS OF VARIANCE FOR BLOOD SUGAR

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F
Drug	9,262.58	3	3,087.53	0.824
Blocking	1,121.59	1	1,121.59	0.299
Interaction	2,323.21	3	744.40	0.207
Error	123,647.02	33	3,746.88	...

dependent on a similar change in the blood sugar. This is felt to be evidence in support of the original postulation, that the block produced must be one in Cori's cycle, preventing a rapid return of the lactic acid produced to liver glycogen.

### SUMMARY

1 Infusions of the four amines, epinephrine, ethylnorepinephrine, Win-3046, and isoproterenol in dogs produce large and significant increases in both blood sugar and lactic acid with or without the blocking agent.

2 When infusions are made after a ganglionic block produced by mecamylamine, the increase of blood sugar is variable but the production of lactic acid is increased significantly by two of the amines, Win-3046 and isoproterenol, over that seen without the block.

3 If the lactic acid increases after blocking are placed in order of magnitude, we find that the four amines are thus arranged in the order of their  $\beta$ -stimulating properties.

4. Evidence is presented here that the conversion of lactic acid to glycogen in the liver is blocked or inhibited by the use of the ganglion blocking agent, mecamylamine, accounting for an accumulation of blood lactic acid under the conditions of the experiment.

### REFERENCES

- (1) Mohme-Lundholm, E., *Acta Physiol Scand Suppl* 29, 108(1953)
- (2) Lundholm, L., *ibid*, 19, 67(1949)
- (3) Deal, C P., and Green, H D., *Circulation Research* 2, 148(1954)
- (4) Cori, C F., and Buchwald, K W., *Am. J. Physiol* 95, 71(1930)
- (5) Starling, E H., "Principles of Human Physiology," 12th ed., Lea & Febiger, Philadelphia, Pa., 1936, pp 148-152
- (6) Komrad, E L., and Loew, E R., *Am J. Phys*, 165, 66(1951)
- (7) Nickerson, M., and Nomaguchi, G M., *J Pharm Exptl Therap*, 107, 284(1953)
- (8) Harvey, S C., and Nickerson, M., *ibid*, 104, 363(1952)
- (9) Cori, C F., *Harvey Lectures*, 41, 253(1946)
- (10) Nelson, N., *J Biol. Chem*, 153, 375(1944)
- (11) Barker, S B., and Summerson, W H., *ibid*, 138, 535(1941)
- (12) Van Slyke, D D., and Hawkins, J A., *ibid*, 79, 739(1928)
- (13) Ahlquist, R P., *J Appl Physiol*, 5, 48(1952)
- (14) McCutcheon, R S., and Ahlquist, R P., *This Journal* 48, 647(1950)
- (15) Ahlquist, R P., *Am J Physiol*, 3, 153(1948)

## Evaluation and Technology of an Emollient Suppository Base\*

By H. I. SILVERMAN

A requisite in the medicinal treatment of local conditions involving the anorectal area is for an emollient, nongreasy, protective application that would favor a sustained release of incorporated medicinals in addition to maintaining the mucous epithelial lining of the area in its normal hydrated condition. Utilizing Lantrol, a non-sensitizing liquid fraction of wool fat, as the major component, a new suppository base has been formulated which possesses, in addition to the foregoing attributes, a high degree of pharmaceutical elegance, adequate plastic range, and is compatible with the great majority of pharmaceuticals commonly incorporated in suppositories. Techniques utilized to determine rate of pharmaceutical release show a desirable prolonged release of medication. Sensitivity testing has also been accomplished. While the base itself is not water soluble it is dispersible in body fluids, and warm water, forming a water-in-oil emulsion that spreads smoothly over the skin forming a protective, partially occlusive, nongreasy film. No difficulties were noted in manufacture, the base sets rapidly and stabilizes quickly.

IN RECENT YEARS a great deal of consideration has been afforded suppositories from the standpoint of base formulation (1-5). Few studies, however, have been devoted to the development of a base having a therapeutic activity of its own in addition to being a vehicle for various types of medication.

\* Received August 13, 1959, from the Research Institute of the Brooklyn College of Pharmacy, Long Island University, Brooklyn, N. Y.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

The great majority of the available vehicle bases melt, disintegrate, or dissolve rapidly at body temperature. In this way medicinal agents are expected to be rapidly absorbed should systemic effects be desired. However, these bases are not especially suited for treating local conditions involving the anorectal area. The widely used fatty bases such as cacao butter and its inexpensive substitutes, the hydrogenated fats, are prone to allow leakage from the anal-

sphincter while not holding medication *in situ* over extended periods of time. In addition, their low melting point (6) is somewhat unsuited for suppositories that are produced on a commercial scale since they produce numerous problems during their manufacture, storage, shipping, and handling. The water-soluble and emulsified bases are preferable to the oleaginous since they permit increased diffusion of medication. Ingredient release, however, may be extremely rapid so that a desirable prolonged and steady medicament release, a requisite in treating anorectal inflammations, does not occur. Suppositories formed from various synthetic gums, natural colloids, and protein materials are not especially suited since they are hydrophilic, tend to swell, and may induce the defecation reflex.

With these considerations in mind, studies were channeled toward the development of a suppository that would: (a) be serviceable as a vehicle for medication commonly incorporated in this dosage form; (b) be stable under normal storage conditions over an extended time; (c) involve no special precautions or procedures during manufacture and eliminate the problems of chipping, fissuring, and splitting; (d) not require a mold lubricant, solidify rapidly (high setting point), and eject easily, and (e) be non-irritating, not leak from the rectum, and able to be handled without deformation.

In addition, the base should be emollient, non-greasy, protective, and allow for a sustained release of incorporated medicinals while maintaining the mucous epithelial lining of the anorectal area in its normal hydrated condition. Since the introduction of wool fat and its derivatives into medicine many references attest to the value of this animal wax as an emollient and adhesive application (7-9). However, due to some manifestations of eczematous hypersensitivity (10) its utilization in dermatology must be with caution to avoid exciting an allergic response in sensitive individuals.

Since the sensitizing component can be demonstrated to be present in the alcohol fraction (11) removal of this constituent by fractionation or chemical alteration such as acetylation results in a hypoallergenic, medicinally and cosmetically acceptable lanolin. Lantrol<sup>1</sup> (12), a liquid fraction of wool grease, was chosen as the major component of this new suppository base. Utilization of a nonsensitizing lanolin for local application should have therapeutic value as wool fat has been demonstrated to enhance the mech-

anism for rehydration of the skin (13), to provide a protective, partially occlusive smooth film, and to be miscible with water forming a water-in-oil emulsion which, in view of its biological origin, may be somewhat analogous to human sebum (9, 10).

Other materials utilized in formulation as thickening and molding agents were the following: Myverol 18-00,<sup>2</sup> a chemical isolation product of hydrogenated lard, high in monoester content obtained by high vacuum molecular distillation and Weecobee base S,<sup>3</sup> a partially hydrogenated oxidatively stable fat which may be utilized as a substitute for cacao butter.

## EXPERIMENTAL

**Formulation and Physical Data.**—In the screening to evolve a suitable suppository vehicle, 75 raw materials were utilized for the preparation of over 50 experimental bases. Of those studied, a base having the following composition was selected: Lantrol, 40%; Weecobee base S, 40%; Myverol 18-00, 20% by weight. The ingredients were combined by fusion at approximately 80-85° with stirring to produce a clear golden-yellow liquid. Molding was accomplished by pouring the fused base at its cloud point of 55° (50-55°) into previously chilled suppository molds. After chilling at 0° for thirty minutes the suppositories were ejected from the mold and stored at 5° for five days in order to allow the isomeric components to assume stable crystalline patterns. The suppositories were then subdivided for the stability studies. Suppositories in dual groups of twenty-five (one group wrapped in aluminum foil, the other with no protective covering) were stored in a refrigerator, at room temperature (20-25°), and on a window ledge exposed to sunlight.<sup>4</sup> After three months each suppository was carefully examined for any evidence of physicochemical change. None in the entire group of one hundred and fifty suppositories showed any evidence of color change, blooming, or deformation.

The softening point of the suppository base was carried out utilizing the ring and ball apparatus of the A S T M and found to fall within the range of 47-48°. A suppository slice was packed into the center of the metallic ring with the ball situated on top of the suppository segment. The apparatus was then immersed in a water bath at a temperature of 25° which was heated by a hot plate equipped with a magnetic stirrer to maintain an even temperature. The rate of temperature rise was regulated at 1° per minute. The softening point range being read when the metallic ball fell free from the metallic ring. Three separate determinations were performed on newly molded lots and on the variously stored samples after the three month aging period. In no case was there any variation from the aforementioned softening point range.

<sup>2</sup> Supplied by Distillation Products Industries.

<sup>3</sup> Supplied by E. F. Drew Co.

<sup>4</sup> This latter group was placed in a specially constructed container, of window glass, with the environmental temperature controlled so as not to rise above 35°.

<sup>1</sup> Supplied by Malmstrom Chemical Corp.



The pH of a dispersion of the base in distilled water was found to be 5.6. This was determined by mixing 5 Gm of the base with 100 ml of distilled water for several minutes, filtering, and reading the pH by means of a Beckman Zeromatic pH meter.

Consistency was determined with a Universal model precision penetrometer. A 15 square cm east of the base was utilized for the penetration. The average of five readings (corners and center) taken with the penetrometer needle was 16.08 mm.

The specific gravity of the suppository base was found to be 0.93.

**Determination of Medicinal Release.**—A colorimetric and a microbiological study was accomplished to ascertain the ability of the base to release incorporated medicinals.

The colorimetric method was performed simultaneously with the Lantrol base and a cacao butter base. Molded and aged suppositories containing known amounts of FD&C Red No. 2 were placed in constant temperature water baths, equipped with magnetic stirrers, set at the softening points of the bases in question. At various intervals aliquot samples were removed, filtered with sintered glass

funnels, and the concentration of dye, as released over the time period of study, determined with a Coleman Jr spectrophotometer at 525 m $\mu$ . Using the data obtained graphs were plotted (Fig 1) to compare the percentage release of dye from the two bases at varying time intervals. The procedure followed is similar to one originally outlined by Gross and Becker (2).

Antibacterial activity in regard to the effectiveness of release of certain selected medicinals from the Lantrol base as compared to a cacao butter base (containing the same ingredients in exactly the same proportion) was determined using four test organisms, Table I. The method followed was to place a freshly sliced segment of a suppository cone containing the medicinal in question, 10 mm in diameter and 10 mm in thickness, upon seeded agar plates. To avoid softening and creeping at the generally accepted incubation temperature of 37° the plates were allowed to remain at room temperature (20–25°) for forty eight hours before being read. Zones of inhibition were measured from the periphery of the suppository segment to the limit of the clear area, the size of the zone being directly related to the ability of the base to release incorporated materials.

**Sensitivity Studies.**—To determine any possible evidence of sensitivity to the base a modified repeat insult patch technique was performed using a pressure type of patch application (14–17). The method used takes into account the possibility of fatiguing of the skin's defense mechanism while also serving to demonstrate possible sensitization build up over a time lapse.

An interracial group of 60 human subjects were used for the experimental study consisting of 55 males and 5 females, ages ranging from 18–30. Approximately 350–500 mg of the test base was applied during each twenty-four-hour testing period. Subjects were instructed to apply the test material to the underside (ventral) of the forearm, midway between the elbow and wrist. The test area covered approximately 10 sq mm of the subject's skin. The materials used in applying the base consisted of a felt pad, doughnut shaped, whose center opening was filled with the base. The pad and base were placed on the skin, covered with a suitable gauze pad, and firmly affixed with adhesive to the skin. Subjects were instructed to apply the patch for twenty four hours, remove patch, wash area, and rest twenty four hours, apply again for twenty four hours, remove patch, wash area, and rest twenty four hours, etc., until five patch applications

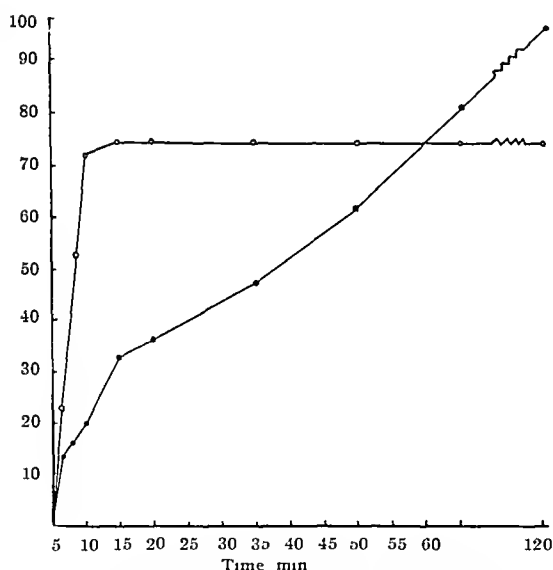


Fig 1—Dye release from suppository bases as a function of time. O, Theobroma base plotted at 37°, ●, Lantrol base plotted at 47°.

TABLE I—ZONES OF INHIBITION

Medicament	Concn in Suppository	Base	Test Organism			
			<i>Proteus Morganii</i> mm	<i>Klebsiella pneumoniae</i> mm	<i>Micrococcus pyogenes citreus</i> mm	<i>Micrococcus pyogenes albus</i> mm
Penicillin G pot	200,000 u	Lantrol	5	3	15	14
		Theobroma			7	4
Tetracycline	100 mg	Lantrol	10	10	13	19
		Theobroma	10	7	10	14
Nitrofurazone	0.2%	Lantrol	5		3	10
		Theobroma	2			
As in No 37, Table III	As in No 37, Table III	Lantrol		5	3	9
		Theobroma	Growth stimulated	Growth stimulated		

TABLE II — SENSITIVITY TESTS

Subject No	Sex	Medical History <sup>a</sup>	Age, yr.	Conclusions
1	M		21	Negative
2	M	"Hay fever"	19	Negative
3	M	Severe acne	19	Slight pruritis first 24 hr. only, negative thereafter
4	M		20	Negative
5	M		19	Negative
6	M		18	Negative
7	M	"Hay fever"	18	Negative
8	M		20	Negative
9	M		19	Negative
10	M		22	Negative
11	M		19	Negative
12	M		23	Negative
13	M		19	Negative
14	M	"Hay fever"	19	Negative
15	M		19	Slight pruritis for first few minutes of first application
16	M		20	Negative
17	M		21	Negative
18	M	Acne	20	Negative
19	F	Food allergies	18	Negative
20	M	"Hay fever"	19	Negative
21	M		28	Negative
22	F	Asthma	26	Negative
23	F		18	Negative
24	M		19	Negative
25	M		21	Negative
26	M	"Hay fever"	26	Negative
27	M	"Hay fever"	30	Negative
28	F		29	Negative
29	M		21	Negative
30	M		27	Negative
31	M		20	2-mm Wheal at end of first application only
32	M		19	Negative
33	M		20	Negative
34	M	"Summer rash"	19	Negative
35	M		19	Negative
36	M	Warts on legs	19	Negative
37	M		20	Negative
38	M	"Hay fever"	19	Negative
39	M		20	Negative
40	M	Severe acne	19	Negative
41	M		19	Negative
42	M		19	Negative
43	M	Food allergies	22	Negative
44	M		20	Negative
45	M	"Hay fever"	19	Negative
46	M		20	Negative
47	M		19	Negative
48	M	Impetigo	19	Negative
49	M		19	Negative
50	M		20	Negative
51	M		20	Negative
52	M		19	Negative
53	M		19	Negative
54	M		20	Negative
55	M		20	Negative
56	M		19	Negative
57	M		19	Negative
58	M	"Hay fever"	19	Negative
59	M	"Hay fever"	19	Negative
60	F		20	Negative

<sup>a</sup> "Hay fever," asthma, gastrointestinal disturbances, urticaria, serum sickness, dermatitic diseases etc

had been completed. After a resting period of thirty days, a second series of five patch applications were completed

Subjects were instructed to record any erythema,

pruritus, tingling, eruptions, or any other abnormalities during the test periods. Table II records subject data, including medical histories, and observations on the sensitivity tests.

TABLE III.—COMPATIBILITY STUDIES

No.	Medication	Amt per Suppos.	Flux Temp., °C.	After 3 Months Storage <sup>a</sup> At RT	Refrigerator
1	Aminophylline	0.5 Gm.	75	C	A
2	Aspirin	0.5 Gm.	65	A	A
3	Argyrol	0.3 Gm.	60	A	A
4	Belladonna ext.	15 mg.	60	A	A
5	Benzocaine	0.15 Gm.	60	C	A
6	Bismuth subcarb	0.2 Gm.	70	A	A
7	Bismuth subgallate	0.2 Gm.	70	A	A
8	Boric acid	0.15 Gm.	60	A	A
9	Butabarbital	65 mg.	70	A	A
10	Stilbestrol	5 mg.	65	A	A
11	Ephedrine sulf	5 mg.	65	A	A
12	Ichthamol	0.3 Gm.	60	D	D
13	Mercurochrome	0.2 Gm.	70	A	A
14	Nitrofurazone	0.2 Gm.	65	A	A
15	Pentobarb sod.	65 mg.	70	A	A
16	Peruvian balsam	0.2 Gm.	50	D	A
17	Phenobarbital	65 mg.	70	A	A
18	Succinylsulfathiazole	0.5 Gm.	60	A	A
19	Sulfanethoxypyridazine	0.25 Gm.	65	A	A
20	Tannic acid	0.3 Gm.	70	A	A
21	Vioform	3.0%	65	A	A
22	Zinc oxide	0.2 Gm.	60	A	A
23	Chloramphenicol	100 mg.	60	A	A
24	Chlortetracycline	100 mg.	60	A	A
25	Erythromycin	100 mg.	60	A	A
26	Neomycin sulf	10 mg.	60	A	A
27	Oxytetracycline	100 mg.	60	A	A
28	Penicillin G pot	65 mg.	60	A	A
29	Polymyxin B sulf.	10 mg.	60	A	A
30	Streptomycin	200 mg.	60	A	A
31	Tetracycline	100 mg.	60	A	A
32	Cortisone acetate	20 mg.	65	A	A
33	Dexamethasone	1 mg.	65	A	A
34	Hydrocortisone acetate	20 mg.	65	A	A
35	Prednisone	5 mg.		A	A
36	Benzocaine	120 mg.		B	A
37	Ephedrine hyd.	5 mg.			
	Vioform	15 mg.	60		
	Zinc oxide	150 mg.			
	Peruvian balsam	150 mg.			
	Belladonna ext.	15 mg.			
	Ephedrine sulf.	3 mg.			
	Zinc oxide	100 mg.	60	A	A
	Boric acid	100 mg.			
	Bismuth subcarb.	100 mg.			
	Peruvian balsam	100 mg.			

<sup>a</sup> A, no change, satisfactory; B, blooming; C, color change; D, softening or deformation.

**Compatibility Tests.**—Several pharmaceuticals<sup>5</sup> which are commonly incorporated in suppository formulations were screened in the evaluation of the Lantrol base as a vehicle for medication. Materials listed (Table III) were, if in the form of a dry, free flowing powder, screened to pass through a number 200-mesh sieve prior to incorporation. Percentages selected were based on those generally found in commercial practice. Incorporation of ingredients into the base was by fusion into the melted base at a temperature found to be optimum for the material in question (Table III), and was accomplished by utilizing a hot plate equipped with a magnetic stirring device. The individual mixtures were stirred continuously while being poured into previously-chilled suppository molds. All molded

suppositories were then aged for five days at 5°. At the completion of the initial cold storage those suppositories containing similar materials were divided into groups of twenty, with one group being wrapped in aluminum foil and the other having no protective covering. All molded suppositories, wrapped and unwrapped, were then placed in storage for a period of three months, both in a refrigerator and at room temperature. At the expiration of this time the suppositories were carefully examined to detect any evidence of incompatibility with the incorporated medicinals. Suppositories were checked for color changes, odor, blooming, cracking, softening, deformation, and spotting. Analytical determinations were not attempted during this portion of the investigation.

<sup>5</sup> The following companies supplied pharmaceuticals utilized in the compatibility investigation: American Cyanamid Co., Burroughs Wellcome & Co., Eaton Laboratories, Eli Lilly & Co., Merck Sharp & Dohme Research Laboratories, Pfizer Laboratories, Upjohn Co.

## DISCUSSION AND CONCLUSIONS

In view of the fact that wool fat is generally accepted as the emollient of choice for medicinal pur-

poses it appeared feasible that it would be of utility, when properly formulated, into an emollient suppository vehicle. In addition to its value as a softening and lubricating agent it also has an affinity for the skin which differentiates it from other similarly used materials. Due to this adhesive property it should be far more suitable (7) to prolong the effects of incorporated medication than other fats or oils. Figure 1 and Table I attest to the value of the Lantrol base regarding release of incorporated material. In addition to a prolonged, sustained action, just short of the entire amount of incorporated dye was detected at the end of a two-hour testing period. While the base itself is not water soluble it is dispersible in both body fluids and warm water forming a w/o emulsion which spreads out smoothly over the skin forming a protective, partially occlusive, nongreasy film.

In comparison the cocoa butter base released the dye very quickly and, since it lacks the coherent property of lanolin, could not be expected to maintain the medication in close contact with the surrounding tissue. Actually, leakage and corresponding loss of medication may be a problem. In addition, it can be noted that there is almost a 30% greater release of dye from the Lantrol base than the theobroma base. Since stability and resistance to deformation are important from a commercial standpoint the suppositories were formulated so as to have a softening point analogous to proprietary products (18).

Also of value from a manufacturing standpoint is the high suspending power of the base for materials with a high density, settling of incorporated medicinals during the mold pouring stage is thusly reduced to a minimum.

The lack of irritation in the group of subjects employed for the sensitivity studies (Table II) illustrates that this base is nonallergenic.

In view of the relatively few abnormalities detected in the compounded lots (Table III) it may be

concluded that the great majority of pharmaceuticals are compatible, save, as might be expected, resinous type products for which the base could be slightly modified. Benzocaine appears too complex with the base in view of the fact that both numbers 5 and 36 of the compounded series showed evidence of abnormalities (Table III). The color change or blooming, which occurred in some samples, was detected only in the unwrapped suppositories, those enclosed in aluminum foil exhibited no evidence of color change or blooming.

In view of the accumulated data it would appear that the proposed Lantrol suppository base has a high degree of pharmaceutical elegance, excellent stability, adequate plastic range, and is compatible with the great majority of pharmaceuticals commonly incorporated in this dosage form.

## REFERENCES

- (1) Whitworth, C. W., and LaRocca, J. P., *THIS JOURNAL*, 48, 353 (1959)
- (2) Gross, H. M., and Becker, C. H., *ibid.*, 42, 96 (1953)
- (3) Gross, H. M., and Becker, C. H., *ibid.*, 42, 498 (1953)
- (4) Ward, W. C., *ibid.*, 39, 265 (1950)
- (5) Collins, A. P., Hohmann, J. R., and Zopf, L. C., *Am. Profess. Pharmacists*, 23, 231 (1957)
- (6) Gross, H. M., and Becker, C. H., *THIS JOURNAL*, 42, 90 (1953)
- (7) Sollmann, T., "A Manual of Pharmacology," 8th ed., W. B. Saunders, Philadelphia, Pa., 1957, p. 124
- (8) Krantz, J. C., and Carr, C. J., "The Pharmacological Principles of Medicinal Practice," 3rd ed., Williams & Wilkins Co., Baltimore, Md., 1954, p. 292
- (9) Barnett, G., *Drug & Cosmetic Ind.*, 83, 292 (1958)
- (10) Sulzburger, M. B., Warshaw, T., and Herrmann, F., *J. Invest. Dermatol.*, 20, 33 (1953)
- (11) Warshaw, T. G., *J. Soc. Cosmetic Chemists*, 4, 290 (1953)
- (12) Sunde, C. J., *U. S. Pat.* 2,758,125 (1956)
- (13) Powers, D. H., and Fox, C., *Proc. Sci. Sect. Toilet Goods Assoc.*, 28, 21 (1957)
- (14) Shelanski, H. A., and Shelanski, M. V., *ibid.*, 19, 5 (1953)
- (15) Jacobi, O., *Drug & Cosmetic Ind.*, 81, 754 (1957)
- (16) Davidow, B., *ibid.*, 80, 608 (1957)
- (17) Sagarin, E., "Cosmetics Science and Technology," Interscience Publishers Inc., New York, N. Y., 1957, pp. 1244-1261.
- (18) Hurtman, C. W., and LaRocca, J. P., *THIS JOURNAL*, 54, 2 (1956).

# Determination of Hydrochlorothiazide in Urine\*

By HERBERT SHEPPARD, THOMAS F. MOWLES, and ALBERT J. PLUMMER

Hydrochlorothiazide may be determined in urine by extracting with ethyl acetate and either reacting directly with chromotropic acid or hydrolyzing first with alkali followed by diazotization and condensation with *N*-(1-naphthyl)-ethylene diamine hydrochloride.

THE RECENT introduction of the new oral diuretic, hydrochlorothiazide<sup>1</sup> (6-chloro-7-sulfamyl-3,4-dihydro-2*H*-1,2,4-benzothiadiazine 1,1-dioxide), which is several-fold more potent than the parent compound chlorothiazide, has necessitated the development of more sensitive and specific methods of analysis. Baer, *et al.* (1), recently described the use of the Bratton-Marshall reaction on diluted urine which had been first hydrolyzed with alkali. Unfortunately, this method may not satisfactorily be applied to the determination of hydrochlorothiazide. The use of much smaller doses of this diuretic does not permit one to make the dilutions called for in the Bratton-Marshall reaction. With smaller dilutions, however, interference is encountered from unknown components in the urine as observed earlier by Marshall, *et al.* (2). This interference can be corrected for by adding two to three different levels of the drug to additional aliquots of the same urine sample and using the values obtained as a standard curve. This method, however, suffers from a lack of sensitivity. The following sections describe a method of separating the drug from interfering substances and the development of a new method which is more specific in that it becomes possible to distinguish hydrochlorothiazide from chlorothiazide and conjugated sulfonamides.

## MATERIALS AND METHODS

**Preparation of Urine Extracts.**—One milliliter of urine is diluted with 2 ml. of distilled water and extracted twice with 18 ml. of ethyl acetate. The pooled ethyl acetate extracts are evaporated to dryness *in vacuo* at 45°.

**Method A.**—Add 1 ml. of 5 *N* NaOH to the dried residue and place in boiling water bath for thirty minutes. Cool and dilute with 8 ml. of distilled water. Acidify with 1 ml. of concentrated HCl. Add 1 ml. of 0.1% aqueous solution of sodium nitrite, mix, and let stand four minutes. Add 1 ml. of 0.5% aqueous solution of ammonium sulfamate,

mix, and let stand three minutes. Finally add 1 ml. of 0.1% aqueous solution of *N*-(1-naphthyl)-ethylene diamine dihydrochloride and within thirty seconds to one minute read the absorbance in the Klett-Summerson colorimeter fixed with a No. 54 filter.

**Method B.**—To the residue from the ethyl acetate extract of the urine is added 5 ml. of acetone along the sides of the tubes. Not all of the material may dissolve so that centrifugation may be necessary. At this point 0.5 ml. of acetone is removed and evaporated to dryness. To the dried residue is added 1 ml. of 15 *M* H<sub>2</sub>SO<sub>4</sub> containing 2 mg./ml. of chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulfonic acid). Heat for five minutes in a boiling water bath, cool, and read color at 570 *mμ*.

For both methods standard curves are prepared by adding 20–100 mcg. of drug to control urine samples and carrying them through the analysis. The standard solutions are prepared by dissolving hydrochlorothiazide in acetone at a concentration of 1 mg./ml. The appropriate aliquot is placed in an empty tube, the acetone evaporated off, and the urine sample added.

## RESULTS

In preliminary work using hydrochlorothiazide labeled with tritium it was observed that the ethyl acetate extracts the drug quantitatively from the urine. This effectively separates the drug from most of the interfering substances present. As observed in Table I, hydrochlorothiazide may be readily hydrolyzed in five minutes by strong acid or alkali with the degree of hydrolysis decreasing with decreasing normality. Since the use of acid hydrolysis gives a lower color intensity, alkaline hydrolysis was resorted to prior to doing the Bratton-Marshall reaction. In Fig. 1 it is observed that time as well as concentration of alkali are important factors in effecting a greater degree of hydrolysis. After

TABLE I.—EFFECT OF NORMALITY OF ACID AND ALKALI ON EXTENT OF HYDROLYSIS OF HYDROCHLOROTHIAZIDE

Reagent	Normality	Relative Degree of Hydrolysis, 5 Min. <sup>a</sup>
H <sub>2</sub> SO <sub>4</sub>	6.00	100
	3.00	83
	1.50	62
	0.75	42
	0.38	24
	0.19	13
NaOH	10.00	100
	5.00	85
	2.50	46
	1.25	17
	0.60	12
	0.30	7
	0.15	3

\* Received October 12, 1959, from the Research Department, Ciba Pharmaceutical Products Inc., Summit N. J.

A preliminary report of this work was presented at the 135th Meeting of the American Chemical Society, Boston, Mass., April 1959.

<sup>1</sup> Ciba's trademark for hydrochlorothiazide is Esidrix.

<sup>a</sup> These values are arbitrarily set at 100 and do not necessarily represent complete hydrolysis.

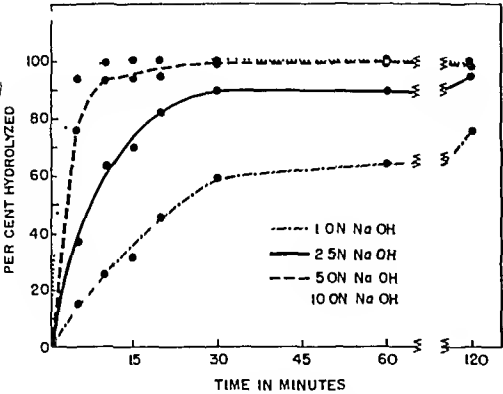


Fig 1—Effect of alkalinity on the degree of hydrolysis of hydrochlorothiazide. Maximum color development under these test conditions is taken as 100% hydrolysis. 200 mcg hydrochlorothiazide in 2 ml solution at 100°

coupling with the N(1-naphthyl)-ethylene diamine there is a tendency for some urine samples to increase in intensity of color. This is due to the presence of interfering substances which are not completely eliminated by extraction with ethyl acetate. It is therefore advisable to read the samples within one minute, at which time color development with the drug alone is complete and remains stable for at least fifteen minutes.

The reaction with chromotropic acid is very rapid, with maximum color being reached within five minutes and remaining stable for at least several hours. The use of acetone to extract the ethyl acetate residues is necessary to remove interfering substances further. Table II compares the chromotropic acid reaction with the Bratton-Marshall reaction on the urines from some dogs which had

TABLE II—COMPARISON OF CHROMOTROPIC ACID AND BRATTON-MARSHALL REACTIONS FOR HYDROCHLOROTHIAZIDE IN DOG URINE

Dog No	Collection Period, hr	Hydrochlorothiazide, mcg / ml of Urine	
		Bratton Marshall	Chromotropic Acid
59	0-2	157	162
	2-4	68	66
	4-6	57	46
	6-7 4	72	64
	7 4-24	20	26
25	0-2	115	118
	2-4	60	54
	4-6	55	
	6-7 4	54	52
	7 4-24	20	22

received hydrochlorothiazide orally. It is observed that the two methods give essentially identical results.

The ethyl acetate extracts may be subjected to paper chromatography to add greater specificity to the method. With butanol saturated with 0.1 N NH<sub>4</sub>OH as the solvent, hydrochlorothiazide has an R<sub>f</sub> of 0.62 by the descending method. The spots may be visualized with short wavelength ultra-violet light (265 mμ) and eluted from the paper with acetone. After evaporation of the acetone, the chromotropic acid reaction may be carried out.

DISCUSSION

Hydrochlorothiazide and chlorothiazide are hydrolyzed by acid or alkali to yield 4-amino-6-chlorobenzene-1,3-disulfonamide. In the Bratton-Marshall reaction the 4-amino group is diazotized with nitrous acid and then coupled with N(1-naphthyl) ethylene diamine. One way to distinguish the two drugs is by varying the time of hydrolysis. Chlorothiazide gives maximum color after about five minutes, whereas the dihydro derivative requires at least thirty minutes. The possible use of sulfonamides along with these diuretics, however, would result in unusually high values.

The chromotropic acid reaction readily differentiates between the two benzothiadiazine compounds. In addition to the disulfonamide, chlorothiazide and hydrochlorothiazide yield formic acid and formaldehyde, respectively, on hydrolysis with acid. The formaldehyde but not the formic acid will then react with the chromotropic acid to give the characteristic purple color. This high degree of specificity plus sensitivity makes the reaction with chromotropic acid the preferred method for the analysis of hydrochlorothiazide.

The sensitivity of both methods is of the order of 10 mcg / ml of urine when carried out as described.

SUMMARY

The application of the Bratton-Marshall reaction to the determination of hydrochlorothiazide has been described.

Another method, incorporating a high degree of specificity, involves the reaction of chromotropic acid with formaldehyde liberated during the acid hydrolysis of hydrochlorothiazide.

REFERENCES

(1) Baer, J. E., Leidy, H. L., Brooks, A. V., and Beyer, K. H., *J. Pharmacol. Exptl. Therap.*, 125, 295(1959).  
(2) Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C., *J. Am. Med. Assoc.*, 103, 953(1937).

# Effect of a Lowered Catalase Level on Actions of Cardiac Glycosides\*

By DUANE G. WENZEL and IVENS A. SIEGEL

Rats were given a high zinc diet to lower body catalase and simultaneously administered 0.5 mg/Kg. digitoxin daily for five weeks. The high zinc diet did not markedly alter the effect of digitoxin on serum or heart sodium or potassium. It did reduce the ECG abnormalities induced by digitoxin. The high zinc diet also reduced the mortality of an acute dose of ouabain for mice ( $P < 0.05$ ) but again did not affect the heart sodium or potassium.

THE OBSERVATION that the unsaturated lactone ring of the cardiac glycosides is essential for the characteristic effects of these agents (1) has led to numerous studies of simple lactone moieties as possible cardiotonic drugs. While certain unsaturated lactones produce systolic standstill in the frog heart (2, 3), their effect on the mammalian heart has been both confirmed (4) and denied (5). The activity of the lactones appears to be related to the formation of a hydroperoxide (6). Hydrogen peroxide itself has been demonstrated to produce similar effects on the rat diaphragm (7) and digitoxin forms a peroxide that is highly effective in the inhibition of enzymes involved in cardiac metabolism (8).

A potentially important enzyme in the biological activity of peroxides is catalase. Among the better known actions of catalase are the decomposition of peroxides and the oxidation of alcohols and aldehydes. The report that the activity of the cardiac glycosides is unrelated to catalase (9) was based on a study conducted on frogs with compounds having questionable activity on the mammalian heart. It was therefore decided to re-examine the possible role of catalase in the functioning of the cardiac glycosides. The approach employed was to determine what, if any, changes in certain activities of the cardiac glycosides would be produced by an *in vivo* reduction of catalase (10).

## EXPERIMENTAL

In order to determine the effect of catalase reduction on the chronic activity of digitoxin in the rat, it was first necessary to approximate a relatively nontoxic chronic dose of the drug. Digitoxin was ad-

ministered in daily intraperitoneal doses of 0.125, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 mg/Kg to two rats per dose. All rats used throughout the study were black hooded females weighing from 170 to 200 Gm. Digitoxin was administered for one week during which time daily ECG records were obtained under pentobarbitalization. Records were obtained with four standard leads by means of an Edin 8122A capacity coupled amplifier and a Brush BL 201 pen writing oscillograph. Because of the rapid heart rate, a paper speed of 125 mm/second was employed.

After seven days one animal in the 1.0 mg/Kg dose group and all animals at larger doses had died. Marked weight loss and electrocardiographic alterations were prominent in these animals. The ECG changes consisted of an increased P-R interval, ST segment depression, and an increase in QRS voltage. As all doses below 1.0 mg/Kg/day produced minimal weight and ECG effects, 0.5 mg/Kg/day of digitoxin was selected as the experimental dose.

Four groups consisting of ten rats per group were employed. One group served as control, the other three groups were placed, respectively, on digitoxin, zinc fortified diet, and zinc plus digitoxin regimens. The zinc fortified diet was prepared by mixing zinc carbonate with coarsely ground Purina laboratory chow to give a final zinc concentration of 0.5%. Electrocardiograms and serum sodium and potassium concentrations were obtained weekly for five weeks. Blood for the latter determination was withdrawn from the jugular vein while the animal was under anesthesia following electrocardiography. A Beckman model 41 direct reading flame photometer was used to determine the serum sodium and potassium. At the end of the fifth week all animals were sacrificed, the hearts removed to determine the sodium and potassium content (11) and the livers removed to determine their catalase content (12).

The liver rather than the heart was analyzed for its catalase content as the liver, kidney, and erythrocytes are the principal locations of this enzyme. Evidence of its presence in other tissues has not only been questionable (13), but we were unable to determine any catalase activity in the rat myocardium. Furthermore, the well known importance of the liver in the functioning of the mammalian heart (14) and the fact that injected digitoxin is quickly and persistently fixed in an active form in the rat liver (15) makes the liver the logical organ in which to determine catalase.

The preceding study was designed to provide information concerning the relationship of catalase to chronic, relatively nontoxic levels of digitoxin. The following procedure was planned to relate the action of catalase to the lethal dose of ouabain. The lethal range of ouabain was first approximated for male albino mice weighing between 30 and 40 Gm. This lethal range was then spanned with seven doses of digitoxin in a geometric progression of 1.5. Ten mice were used for each of the seven

\* Received November 23, 1960 from the University of Kansas School of Pharmacy, Lawrence.

Abstracted from a portion of the data contained in a thesis submitted to the Graduate School of the University of Kansas by Ivens A. Siegel for the degree of Master of Science in Pharmacology.

This investigation was supported by a research grant from the University of Kansas.

doses. Seventy animals were fed the 0.5% zinc diet and an equal number the control diet for five weeks. The calculated doses of ouabain were then administered intraperitoneally to both groups. Sodium and potassium concentrations of all the experimental mouse hearts were determined at the termination of the experiment. The concentrations of these cations were also determined in a group of ten untreated controls.

## RESULTS AND CONCLUSIONS

Electrocardiographic differences were observed between the digitoxin and zinc-digitoxin treated rats. The greatest and most consistent changes were observed in the amplitude of the QRS voltage and in the character of the ST segment. While the control and zinc treated groups displayed negligible alterations in the ECG, most of the digitoxin treated rats displayed increased QRS voltage and a depressed ST segment. A minimal QRS voltage increase and ST depression were seen in a minority of the zinc digitoxin animals.

The mean weekly serum sodium and potassium values are presented in Figs 1 and 2. No significant change in sodium had occurred at five weeks although the sodium level of the digitoxin group had undergone a considerable fall. A marked fall in serum sodium was demonstrated in the zinc and zinc digitoxin groups at the second week of treatment although both recovered much of the sodium loss during the last three weeks. The serum potassium values of the digitoxin groups were all significantly elevated ( $P < 0.01$ ) above the control at the one week period. At the two week period the digitoxin

( $P < 0.01$ ) and zinc digitoxin ( $P < 0.05$ ) were still elevated. At the termination of the experiment, however, all treated groups demonstrated a slightly lowered but remarkably uniform serum potassium content. It is apparent from the graphs that the zinc diet, instead of modifying the effect of digitoxin on the serum sodium and potassium, appeared to influence the concentrations in the same general direction as the digitoxin.

Table I contains the mean heart sodium and potassium concentrations and the mean liver catalase activities. There were no significant changes in sodium and potassium concentrations. In both groups treated with zinc the catalase was reduced to about 60% of the concentration in the control or the digitoxin group. The digitoxin did not affect the catalase levels.

The mortality curves obtained with the ouabain and the zinc ouabain treated mice are given in Fig 3. When analyzed according to the method of Litchfield and Wilcoxon (16), the  $LD_{50}$ 's and 95% confidence limits are 7.5 mg/Kg (5.59-10.05) for ouabain and 11.7 mg/Kg (8.36-16.38) for zinc-ouabain. The difference of the  $LD_{50}$ 's was barely significant at the 0.05% level. The ranges of the mean heart sodium and potassium levels of all doses of the ouabain treated mice were 4.36-5.04 and 7.19-7.98 meq, respectively. The ranges of the sodium and potassium levels for the zinc ouabain hearts were 4.32-4.98 and 7.17-7.47, respectively. Mean values for the ten control animals were 4.62 for sodium and 7.47 meq for potassium. It is apparent that neither the administration of ouabain nor the combined effect of ouabain and a zinc diet caused a significant alteration of the sodium and potassium concentrations. This is in contrast to a rather general agreement that toxic doses of the cardiac glycosides liberate potassium from the heart muscle (17).

The results as a whole are, at best, suggestive and

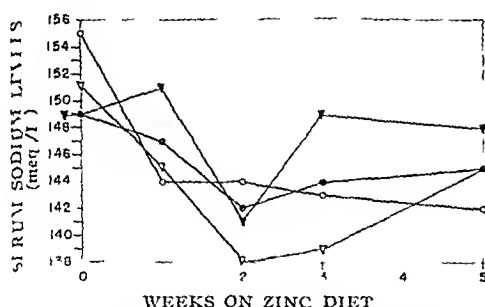


Fig 1—Mean weekly serum sodium values ●, Control, ○, digitoxin, ▼, zinc, ▽, zinc and digitoxin.

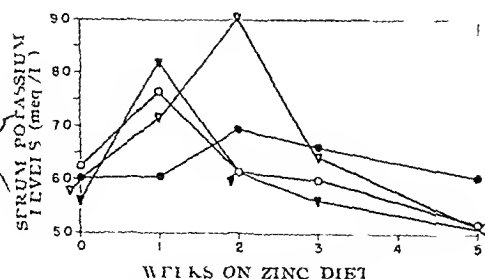


Fig 2—Mean weekly serum potassium values ●, Control, ○, digitoxin, ▼, zinc, ▽, zinc and digitoxin.

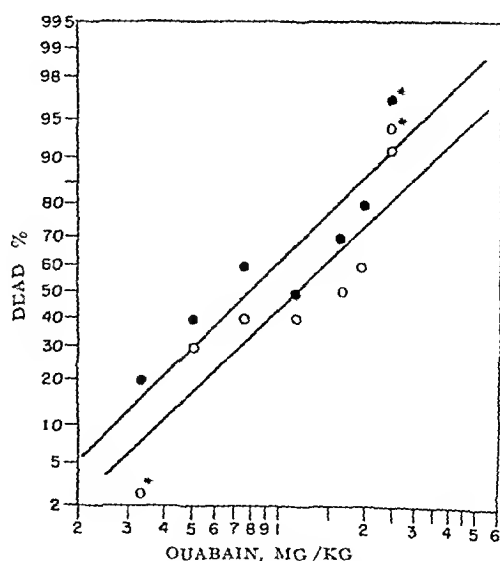


Fig 3—Mortality curves obtained with the ouabain and the zinc-ouabain treated mice ●, Control, ○, zinc diet, \*, calculated points.



TABLE I.—MEAN SODIUM AND POTASSIUM CONTENT OF THE HEARTS AND CATALASE CONTENT OF THE LIVERS OF ZINC- AND DIGITOXIN-TREATED RATS<sup>a</sup>

Determination	Control	Digitoxin	Zinc Diet	Zinc Digitoxin
Heart sodium <sup>b</sup>	5 226 ± 0 359	5 082 ± 0 168	4 464 ± 0 193	4 788 ± 0 678
Heart potassium <sup>b</sup>	7 124 ± 0 389	6 510 ± 0 125	6 410 ± 0 324	6.475 ± 0 225
Liver catalase <sup>c</sup>	0 789 ± 0 0502	0 793 ± 0 0235	0 504 ± 0.0179	0 505 ± 0 0640

<sup>a</sup>  $S E + \sqrt{\frac{\sum d^2}{N(N-1)}}$   
<sup>b</sup> Meq /100 Gm heart, wet weight  
<sup>c</sup> Meq NaBO<sub>3</sub> 4H<sub>2</sub>O/mg liver, wet weight.

do not indicate any appreciable interaction between the cardiac glycosides tested and the catalase level. Even if marked changes in the action of the cardiac glycosides could be produced by a high zinc diet, one could not assume that the changes were a direct consequence of lowered catalase activity as they could be the result of some undefined effect of a zinc diet. With this limitation in mind, there were two results in which the zinc diet seemed to reduce the activity of the cardiac glycosides. It was quite apparent in the tendency to normalize the electrocardiograms of the digitoxin-treated rats. Likewise, the reduction in the mouse-ouabain mortality produced by the zinc diet, although barely significant, was in the same direction at each of the seven doses tested. It is quite possible that the mouse mortality may have been the result of toxicity to the brain as a number of the animals died in convulsive seizures. Rats have been reported to be especially susceptible to the CNS effects of rapidly administered cardiac glycosides (18). It is possible that the change in the mortality may be related in some manner to the facts that the cardiac glycosides stimulate oxygen uptake by the brain (19), the brain has a low catalase content and is susceptible to hydrogen peroxide (20).

SUMMARY

1. The catalase level of female black-hooded rats and male albino mice was lowered by the administration of a diet containing 0.5 per cent zinc.

2. Zinc-fed rats and controls were administered 0.5 mg./Kg./day of digitoxin for five weeks. Electrocardiograms and serum sodium and potassium were determined at weekly intervals. The hearts were analyzed for sodium and potassium and the liver for catalase at the end of the five-week period. The zinc diet re-

duced the rat liver catalase by 40 per cent and decreased the ECG abnormalities induced by digitoxin, but did not significantly alter the effect of digitoxin on serum or heart sodium or potassium.

3. The LD<sub>50</sub> of ouabain was determined in mice treated with zinc for five weeks and in controls. The hearts were analyzed for sodium and potassium at the time of death. Treatment with zinc reduced the mortality of the mice treated with ouabain (*P* < 0.05) but did not affect the sodium or potassium content of the heart.

REFERENCES

(1) Chen, K. K., and Elderfield, R. C., *J. Pharmacol. Exptl. Therap.*, 70, 338 (1940).  
(2) Chen, K. K., Steldt, E. A., Fried, J., and Elderfield, R. C., *ibid.*, 74, 381 (1942).  
(3) Krayer, O., Mendez, R., Moisset de Espanes, E., and Linstead, R. P., *ibid.*, 74, 372 (1942).  
(4) Bennett, D. R., Anderson, K. S., Anderson, M. V., Robertson, D. N., and Chenoweth, M. B., *ibid.*, 122, 489 (1958).  
(5) Walton, R. P., Cotten, M. DeV., and McCord, W. M., *Proc. Soc. Exptl. Biol. Med.*, 74, 548 (1950).  
(6) Wenzel, D. G., and Keplinger, M. L., *This Journal*, 42, 553 (1953).  
(7) Hobbiger, F., *J. Physiol.*, 113, 14 (1951).  
(8) Procter, C. D., Rebar, J., Jr., and Tigerman, B., *Ann. N. Y. Acad. Sci.*, 62, 377 (1955).  
(9) Mendez, R., *J. Pharmacol. Exptl. Therap.*, 81, 151 (1944).  
(10) Sutton, W. R., and Nelson, V. E., *Proc. Soc. Exptl. Biol. Med.*, 36, 211 (1937).  
(11) Robertson, W. van B., and Peyser, P., *Am. J. Physiol.*, 166, 277 (1951).  
(12) Feinstein, R. N., *J. Biol. Chem.*, 180, 1197 (1949).  
(13) Theorell, H., "Enzymes," vol. II, part 1, Academic Press, New York, 1951, p. 402.  
(14) Mertens, O., and Rein, H., *Naturwissenschaften*, 36, 237 (1949).  
(15) Brown, B. T., Shepherd, E. E., and Wright, S. E., *J. Pharmacol. Exptl. Therap.*, 118, 39 (1956).  
(16) Litchfield, J. T., Jr., and Wilcoxon, F., *ibid.*, 96, 99 (1949).  
(17) Rayner, B., and Weatherall, M., *Brit. J. Pharmacol.*, 12, 371 (1957).  
(18) Gold, H., Modell, W., Cattell, McK., Benton, J. G., and Cottle, W., *J. F. P.*, 1, 105 (1957).  
(19) Wollenberger, E., and Dickens, F., *Thomas, Springfield, Ill.*, 1955, p. 642.

# Menispermaceae Alkaloids I\*

## The Alkaloids of *Cissampelos pareira* Linn. and the Origin of *Radix Pareirae Bravae*

By S. MORRIS KUPCHAN, NAOKATA YOKOYAMA, and JACK L. BEAL†

The roots and vines of *Cissampelos pareira* Linn. from Madras yielded *l*-curine, *d*-isochondrodendrine, and hayatin. Preliminary pharmacological study of the methanol-extractable alkaloids, of the methiodide prepared from the latter mixture, and of the quaternary alkaloids, showed that all had curare-like activity. The relationship of the composition and pharmacological activity of the alkaloidal mixture to the problem of the botanical source of the drug *radix pareirae bravae* is discussed.

**C**ISSAMPELOS PAREIRA Linn. is a climbing shrub distributed throughout tropical and subtropical India and warm parts of Asia, East Africa, and America. The roots are reported to have found use as a diuretic, febrifuge, heart trouble remedy, and against dysentery and sores (1).

Wiggers in 1840 (2) isolated an amorphous alkaloid from the roots of a South American *C. pareira* sample, and the name pelosine was assigned to the alkaloid. Scholtz (3, 4) showed that pelosine is identical with *l*-curine (*l*-bebeerine<sup>1</sup>). Bhattacharji, Sharma, and Dhar reported in 1956 (5) that *C. pareira* Linn. from Kashmir yielded two new alkaloids, hayatin and hayatinin, and that the same species from Pilibhit yielded hayatin and *l*-curine, but no hayatinin. The methiodide of hayatin was shown to possess powerful neuromuscular blocking activity comparable to that of *d*-tubocurarine chloride (6).

The present report describes a preliminary study of the alkaloids of the roots and vines of *C. pareira* Linn. from Madras. Coarsely ground plant was extracted successively with petroleum ether, methanol, 1.5 per cent triethylamine in methanol, and 1.5 per cent hydrochloric acid solution. Each extract was processed for alkaloid content by the procedure summarized in Fig. 1. The crude fractions thus obtained were either crystallized directly, or subjected to chromatography on alumina, whereby separation into crystallizable fractions was effected. The isolation procedure afforded *l*-curine as the principal isolable alkaloidal constituent (1.2 per cent), and *d*-isochondrodendrine (0.2 per cent) and hayatin (0.005 per cent) as lesser isolable constituents. It is noteworthy that the plant from

Kashmir yielded more hayatin (0.15 per cent) but no *l*-curine, whereas the sample from Pilibhit yielded *l*-curine (0.33 per cent) but neither hayatinin nor *d*-isochondrodendrine (5). Preliminary pharmacological study of our methanol-extractable alkaloids (fractions A, B, and C), of the methiodide prepared from the latter mixture, and of the quaternary alkaloids (fraction G) showed that all had curare-like activity.<sup>2</sup>

*C. pareira* was regarded for a great many years as the botanical origin of the drug *radix pareirae bravae*. In 1648, Piso (7) and Marcgrav (8) both described and figured a plant with a wide distribution in the New World known as *Caapeba*, which had a reputation in the treatment of calculus and other bladder complaints. The fact that *Caapeba* was used in the treatment of the same ailments for which *pareira brava* was used led to the early presumption that *Caapeba* was the botanical origin of *pareira brava*. Furthermore, Piso's description of the fruit of the *Caapeba* plant closely fitted the fruit of *Cissampelos pareira*. Linnaeus in 1763 gave the name *C. pareira* to *Caapeba* (9) and thereby lent the weight of his authority to the belief that *pareira brava* originated from *C. pareira*. Woodville (10) in 1790 listed *pareira brava* as a synonym for *C. pareira* and spoke of them interchangeably. Loudon (11) in 1841 also stated that *C. pareira* was the botanical origin of *pareira brava*. In four revisions of the United States Pharmacopeia beginning in 1842 (12-15), *C. pareira* Linn. was recognized as the origin of the drug *pareira*, the official name of *pareira brava*.

Hanbury (16) in 1873 reopened the question of the botanical origin of *pareira brava*. He compared samples of the drug from Brazil with authentic specimens of *C. pareira* Linn. from Jamaica, Trinidad, Ceylon, and Brazil and reported that in a histological study the samples of

\* Received January 18, 1960, from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of Wisconsin, Madison.

Supported in part by a grant from the National Heart Institute [H-2952 (C2)].

† National Science Foundation Science Faculty Fellow, 1958-1959. Permanent address: College of Pharmacy, Ohio State University, Columbus.

<sup>1</sup> The name curine is preferable to the ill-chosen term bebeerine. See the discussion by Wintersteiner, O., "Curare and Curare-Like Agents," Elsevier Publishing Co., Amsterdam, 1959, pp. 153-154.

<sup>2</sup> The authors wish to thank Mr. Edward Macko of the Smith Kline and French Laboratories, Philadelphia, Pa., for the pharmacological results reported herein.

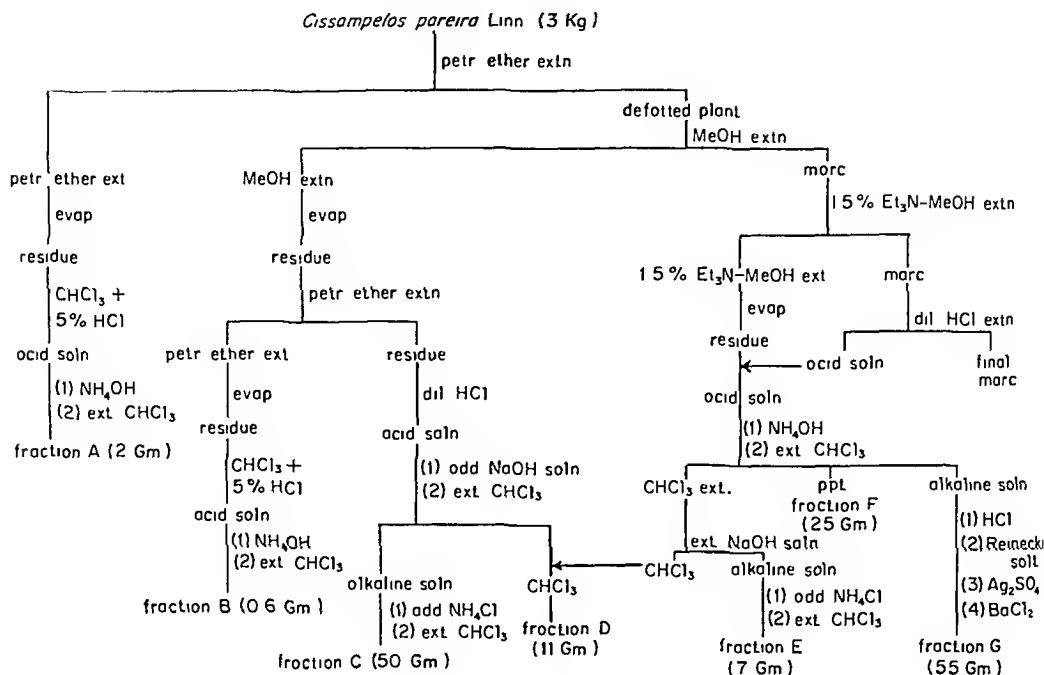


Fig. 1—Flow sheet for separation of alkaloids of *Cissampelos pareira* Linn.

*pareira brava* differed notably from the *Cissampelos* specimens. Hanbury drew the conclusion that the pharmacologically-active *pareira brava* came from *Chondrodendron tomentosum*. He stated, however, that in England a drug called *pareira brava*, completely devoid of medicinal value, was being sold and he claimed that the English drug was *C. pareira*. Hanbury's work appears to have exerted a profound influence on the subject. The sixth revision of the United States Pharmacopeia (17) in 1882 listed *Ch. tomentosum* as the official botanical origin of *pareira*. In addition, many other authoritative publications accepted Hanbury's contention.

More recently, Krukhoff and Moldenke (18) have surveyed the American *Mentha* and concluded that *Ch. platyphyllum* (St. Hil.) Miers is the major source of *pareira*.

In 1899, Scholtz (4) isolated *l*-curine from *pareira brava*. Somewhat later, Scholtz and Koch (27) examined a large quantity of *pareira brava* from a different commercial source and found only traces of *l*-curine. As a result of these recorded variations and of their own experiences, Faltis and Neumann (28) came to the conclusion that *Ch. platyphyllum* (St. Hil.) Miers is the true source of *pareira brava* but that unless it is from closely allied species exist which cannot be differentiated by the pharmacognosist, it is not possible to determine the source of the drug. *Ch. platyphyllum* is subject to elimination of the alkaloid content. The most significant chemical contribution to the field has been that of King in 1940 (29). King studied the alkaloid of *radix pareirae bravae* of the English market, which was botanically identified *Ch. platyphyllum* (St. Hil.) Miers collected in Brazil in the region of Rio de Janeiro and from Bahia, and of *Ch. microphyllum* also collected near Bahia. The root of *Ch. platyphyllum* from Rio de Janeiro contains *l*-curine and *d*-isochondrodendrine, and the same alkaloids were found in the root of the species collected near Bahia and identified as *pareira brava* by a local exporter of medicinal plants. *Ch. microphyllum* from Bahia contained *d*-curine and *d*-isochondrodendrine. *Radix pareirae bravae* of the English market also contained *d*-curine and *d*-isochondrodendrine in addition to a small amount of *l*-isococlaurine. King concluded when *pareira brava* yields *l*-curine it comes from *Ch. platyphyllum* and when it yields *d*-curine, from *Ch. microphyllum*.

It is noteworthy that our study of *C. pareira* Linn. has demonstrated that the principal isolable alkaloids, *l*-curine and *d*-isochondrodendrine, are the same as the principal isolable alkaloids of *Ch. platyphyllum* (St. Hil.) Miers. The determination of the botanical source of *pareira brava* of the present work, and in *pareira brava* is so complicated that it is not possible to determine the source of the drug.

It is noteworthy that our study of *C. pareira* Linn. has demonstrated that the principal isolable alkaloids, *l*-curine and *d*-isochondrodendrine, are the same as the principal isolable alkaloids of *Ch. platyphyllum* (St. Hil.) Miers. The determination of the botanical source of *pareira brava* of the present work, and in *pareira brava* is so complicated that it is not possible to determine the source of the drug.

In the light of our findings concerning the isolable alkaloids and curate like activity of *C. pareira* Linn, the conclusions of Hanbury (16), King (29), and others concerning the lack of medicinal value of the latter plant appear unwarranted. It appears reasonable that medicinally-useful *pareira* may have originated at various times in history from either *Cissampelos pareira* Linn or the *Chondrodendron* species now regarded as the true sources.

## EXPERIMENTAL

Melting points have been corrected for stem exposure. Values of  $[\alpha]_D^{25}$  have been approximated to the nearest degree. Infrared spectra were determined on a Baird double beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Paper chromatography was conducted by the descending technique on Whatman No. 1 paper.

**Extraction of Alkaloids from *Cissampelos pareira*, Separation into Main Fractions**—Coarsely ground *C. pareira*<sup>3</sup> (air dried roots and vines, 3 Kg.) from Madras was extracted continuously for four days with petroleum ether (Shell B, b p 60–80°) in a Soxhlet type extractor. Evaporation under reduced pressure left a semisolid residue (138 Gm). A portion of the residue (10.3 Gm) was dissolved in chloroform (100 cc). The chloroform solution was washed with water (10 cc) and was then extracted with 5% hydrochloric acid (three 50 cc portions). The combined acid extract was washed with ether (three 20 cc portions) and was then made alkaline with ammonium hydroxide. The alkaline solution was extracted repeatedly with chloroform, and the chloroform extracts were combined and concentrated to about 100 cc. The extraction into acid and reconversion to free base was repeated as above. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield 150 mg of fraction A (see Fig. 1).

The dried marc remaining from the petroleum ether extraction was next extracted continuously with methanol, with a fresh charge of solvent at the end of two days. When the extraction was stopped, after five days, the extract returning to the pot yielded upon evaporation, a residue which did not give a positive test with Mayer's reagent. The methanol extract was concentrated under reduced pressure to a brown semisolid residue. This residue was triturated with petroleum ether (500 cc) to remove remaining oil. Workup of this petroleum ether extract as above yielded 610 mg of alkaloid fraction B. The petroleum ether insoluble residue was next triturated with 15% hydrochloric acid (2 L.). The extract was washed with ether (three 500 cc) and made alkaline with ammonium hydroxide. The alkaline solution was extracted with chloroform (5 L.), and the insoluble precipitate which formed was rejected. To separate the phenolic alkaloids, the chloroform solution

was extracted with 1% sodium hydroxide solution (2 L.), and the strongly alkaline extract was washed with chloroform. The alkaline extract was treated with sufficient solid ammonium chloride to bring the pH to about 8.5, whereupon a pale yellow precipitate separated. The solution was extracted exhaustively with chloroform, the chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 50 Gm of brown phenolic alkaloid fraction C. The chloroform washings were combined with the chloroform solution of nonphenolic alkaloids and with a chloroform solution of the corresponding fraction derived by triethylamine-methanol extraction (see below). The combined solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 11 Gm of brown, semisolid, nonphenolic alkaloid fraction D.

The marc remaining from the methanol extraction was next extracted continuously with 15% triethylamine in methanol for six days. When the extraction was stopped, the extract returning to the pot yielded, upon evaporation, a residue which did not give a positive test with Mayer's reagent. The marc remaining from this extraction was percolated with 15% hydrochloric acid (5 L.). The triethylamine-methanol extract was evaporated to dryness under reduced pressure, and the residue was triturated with the hydrochloric acid extract of the marc. The acid solution was washed with chloroform (three 1-L. portions) and was then made alkaline with ammonium hydroxide. The alkaline solution was extracted repeatedly with chloroform and the insoluble precipitate which remained at the interface was collected (fraction F, 25 Gm). The chloroform solution was treated as above to separate a nonphenolic portion (combined into fraction D) and the phenolic alkaloid fraction E (7 Gm). The aqueous layer was combined with all the other aqueous layers obtained during the fractionation procedure, acidified with hydrochloric acid, and treated with a saturated Remecke salt solution. The dried Remecke salt (136 Gm) of the quaternary alkaloids was treated essentially by the method of Tomita and Kikuchi (30) to liberate the quaternary alkaloids. The salt was dissolved in acetone. After filtration of insoluble solids, the acetone solution was treated with saturated aqueous silver sulfate solution to complete precipitation. The silver Remeckate was removed by filtration, and the filtrate was treated with barium chloride solution to complete precipitation. After removal of the barium sulfate by centrifugation, the supernatant solution was evaporated to dryness under reduced pressure to yield 55 Gm of quaternary chloride fraction G. Studies of this fraction will be reported at a later date.

**Isolation of Alkaloids—Fraction A**—Crystallization of the crude alkaloid fraction (150 mg) from methanol afforded needles (108 mg), m p 217–219°,  $[\alpha]_D^{25} -293^\circ$  (c 1.00, ethanol),  $[\alpha]_D^{25} -317^\circ$  (c 1.15, CHCl<sub>3</sub>),  $\lambda_{max}$  280 mμ (ε 9,300), 285 mμ (ε 9,200). The melting point was not depressed on admixture with an authentic sample of l-currine<sup>4</sup>.

<sup>3</sup> C. B. Subochina University, India, for confirming the identity and Co. Madras, India, for plant materials to us.

<sup>4</sup> The authors thank Dr. James D. Dutcher, Squibb Institute for Medical Research, New Brunswick, N. J. for an authentic sample of l-currine.

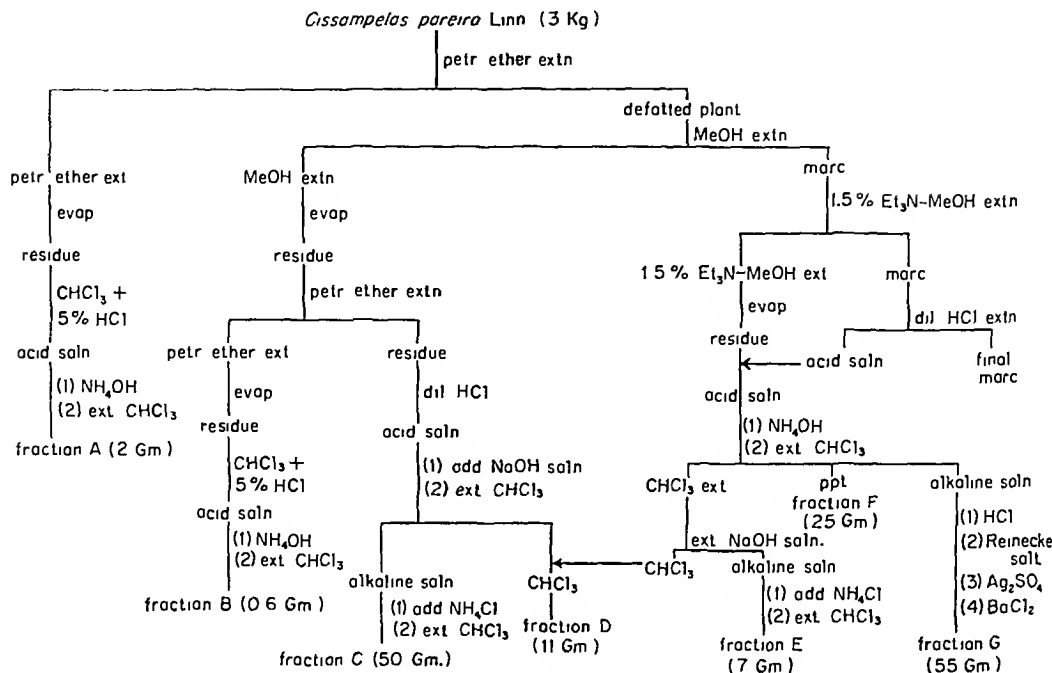


Fig. 1—Flow sheet for separation of alkaloids of *Cissampelos pareira* Linn.

*pareira brava* differed notably from the *Cissampelos* specimens. Hanbury drew the conclusion that the pharmacologically-active *pareira brava* came from *Chondrodendron tomentosum*. He stated, however, that in England a drug called *pareira brava*, completely devoid of medicinal value, was being sold and he claimed that the English drug was *C. pareira*. Hanbury's work appears to have exerted a profound influence on the subject. The sixth revision of the United States Pharmacopeia (17) in 1882 listed *Ch. tomentosum* as the official botanical origin of *pareira*. In addition, many other authoritative publications accepted Hanbury's contention (18-25). More recently, Krukhoff and Moldenke (26) critically surveyed the American *Menispermaceae* and concluded that *Ch. platyphyllum* (St. Hil.) Miers is the major source of *pareira*.

In 1899, Scholtz (4) isolated *l*-curine from *pareira brava*. Somewhat later, Scholtz and Koch (27) examined a large quantity of *pareira brava* from a different commercial source and found only traces of *l*-curine. As a result of these recorded variations and of their own experiences, Faltis and Neumann (28) came to the conclusion that *Ch. platyphyllum* (St. Hil.) Miers is the true source of *pareira brava* but that unless roots from closely allied species exist which cannot be differentiated by the pharmacognosist, it may be that *Ch. platyphyllum* is subject to climatic and seasonal influences which determine the nature

of the alkaloid content. The most significant chemical contribution to the field has been that of King in 1940 (29). King studied the alkaloids of *radix pareirae bravae* of the English market, of botanically identified *Ch. platyphyllum* (St. Hil.) Miers collected in Brazil in the region of Rio de Janeiro and from Bahia, and of *Ch. microphyllum*, also collected near Bahia. The root of *Ch. platyphyllum* from Rio de Janeiro contained *l*-curine and *d*-isochondrodendrine, and the same alkaloids were found in the root of the species collected near Bahia and identified as *pareira brava* by a local exporter of medicinal plants. *Ch. microphyllum* from Bahia contained *d*-curine and *d*-isochondrodendrine. *Radix pareirae bravae* of the English market also contained *d*-curine and *d*-isochondrodendrine in addition to a small amount of *l*-isococlaurine. King concluded when *pareira brava* yields *l*-curine it comes from *Ch. platyphyllum* and when it yields *d*-curine, from *Ch. microphyllum*.

It is noteworthy that our study of *C. pareira* Linn. has demonstrated that the principal isolable alkaloids, *l*-curine and *d*-isochondrodendrine, are the same as the principal isolable alkaloids of *Ch. platyphyllum* (St. Hil.) Miers. The determination of the botanical source of *pareira brava* is beyond the scope of the present work, and indeed, the history of *pareira brava* is so complex that it may now be impossible to determine the original historical botanical source of the drug.

In the light of our findings concerning the isolable alkaloids and curare-like activity of *C. pareira* Linn., the conclusions of Hanbury (16), King (29), and others concerning the lack of medicinal value of the latter plant appear unwarranted. It appears reasonable that medicinally-useful *pareira brava* may have originated at various times in history from either *Cissampelos pareira* Linn. or the *Chondrodendron* species now regarded as the true sources.

## EXPERIMENTAL

Melting points have been corrected for stem exposure. Values of  $[\alpha]_D$  have been approximated to the nearest degree. Infrared spectra were determined on a Baird double beam infrared recording spectrophotometer. Ultraviolet absorption spectra were determined in 95% ethanol on a Cary recording spectrophotometer (model 11 MS). Paper chromatography was conducted by the descending technique on Whatman No. 1 paper.

**Extraction of Alkaloids from *Cissampelos pareira*, Separation into Main Fractions.**—Coarsely ground *C. pareira*<sup>3</sup> (air-dried roots and vines, 3 Kg.) from Madras was extracted continuously for four days with petroleum ether (Skelly B, b. p. 60–80°) in a Soxhlet-type extractor. Evaporation under reduced pressure left a semisolid residue (138 Gm.). A portion of the residue (10.3 Gm.) was dissolved in chloroform (100 cc.). The chloroform solution was washed with water (10 cc.) and was then extracted with 5% hydrochloric acid (three 50-cc. portions). The combined acid extract was washed with ether (three 20-cc. portions) and was then made alkaline with ammonium hydroxide. The alkaline solution was extracted repeatedly with chloroform, and the chloroform extracts were combined and concentrated to about 100 cc. The extraction into acid and reconversion to free base was repeated as above. The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure to yield 150 mg. of fraction A (see Fig. 1).

The dried marc remaining from the petroleum ether extraction was next extracted continuously with methanol, with a fresh charge of solvent at the end of two days. When the extraction was stopped, after five days, the extract returning to the pot yielded upon evaporation, a residue which did not give a positive test with Mayer's reagent. The methanol extract was concentrated under reduced pressure to a brown semisolid residue. This residue was triturated with petroleum ether (500 cc.) to remove remaining oil. Workup of this petroleum ether extract as above yielded 610 mg. of alkaloid fraction B. The petroleum ether-insoluble residue was next triturated with 1.5% hydrochloric acid (2 L.). The acid extract was washed with ether (three 500-cc. portions), made alkaline with ammonium hydroxide, and extracted with chloroform (5 L.); a small quantity of insoluble precipitate which separated at the interface was rejected. To sepa-

rate the phenolic alkaloids, the chloroform solution was extracted with 1% sodium hydroxide solution (2 L.), and the strongly alkaline extract was washed with chloroform. The alkaline extract was treated with sufficient solid ammonium chloride to bring the pH to about 8.5, whereupon a pale yellow precipitate separated. The solution was extracted exhaustively with chloroform; the chloroform extract was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 50 Gm. of brown phenolic alkaloid fraction C. The chloroform washings were combined with the chloroform solution of nonphenolic alkaloids and with a chloroform solution of the corresponding fraction derived by triethylamine-methanol extraction (see below). The combined solution was washed with water, dried over anhydrous sodium sulfate, and evaporated to dryness under reduced pressure to yield 11 Gm. of brown, semisolid, nonphenolic alkaloid fraction D.

The marc remaining from the methanol extraction was next extracted continuously with 1.5% triethylamine in methanol for six days. When the extraction was stopped, the extract returning to the pot yielded, upon evaporation, a residue which did not give a positive test with Mayer's reagent. The marc remaining from this extraction was percolated with 1.5% hydrochloric acid (5 L.). The triethylamine-methanol extract was evaporated to dryness under reduced pressure, and the residue was triturated with the hydrochloric acid extract of the marc. The acid solution was washed with chloroform (three 1-L. portions) and was then made alkaline with ammonium hydroxide. The alkaline solution was extracted repeatedly with chloroform and the insoluble precipitate which remained at the interface was collected (fraction F, 25 Gm.). The chloroform solution was treated as above to separate a nonphenolic portion (combined into fraction D) and the phenolic alkaloid fraction E (7 Gm.). The aqueous layer was combined with all the other aqueous layers obtained during the fractionation procedure, acidified with hydrochloric acid, and treated with a saturated Reinecke salt solution. The dried Reineckate salt (136 Gm.) of the quaternary alkaloids was treated essentially by the method of Tomita and Kikuchi (30) to liberate the quaternary alkaloids. The salt was dissolved in acetone. After filtration of insoluble solids, the acetone solution was treated with saturated aqueous silver sulfate solution to complete precipitation. The silver Reineckate was removed by filtration, and the filtrate was treated with barium chloride solution to complete precipitation. After removal of the barium sulfate by centrifugation, the supernatant solution was evaporated to dryness under reduced pressure to yield 55 Gm. of quaternary chloride fraction G. Studies of this fraction will be reported at a later date.

**Isolation of Alkaloids—Fraction A.**—Crystallization of the crude alkaloid fraction (150 mg.) from methanol afforded needles (108 mg.), m. p. 217–219°;  $[\alpha]_D^{25} = -293^\circ$  (c 1.00, ethanol);  $[\alpha]_D^{26} = -317^\circ$  (c 1.15, CHCl<sub>3</sub>);  $\lambda_{max}^{alc.} = 280 m\mu$  ( $\epsilon$  9,300); 285  $m\mu$  ( $\epsilon$  9,200). The melting point was not depressed on admixture with an authentic sample of *l*-curine.<sup>4</sup>

<sup>3</sup> The authors thank Dr. C. B. Sulochana, University Botany Laboratory, Madras, India, for confirming the identity of the plant, and Rajaranga and Co., Madras, India, for gathering and forwarding the dried plant materials to us.

<sup>4</sup> The authors thank Dr. James D. Dutcher, Squibb Institute for Medical Research, New Brunswick, N. J., for an authentic sample of *l*-curine.

The paper chromatographic behavior<sup>4</sup> and infrared spectrum in chloroform solution were identical with those of the authentic *l*-curine sample.

*Fraction B*—Crystallization of the crude alkaloid fraction (610 mg.) from methanol gave 420 mg. of *l*-curine.

*Fraction C*—Crystallization of the crude alkaloid fraction (50 Gm.) from methanol afforded 41.5 Gm. of a crude microcrystalline mixture, m. p. 209–211°. A portion of the microcrystalline mixture (1.105 Gm.) was chromatographed on Woelm "neutral" alumina (20 Gm.). The alkaloid was applied to the column as a solution in benzene and the column was developed by successive elution with benzene-chloroform, chloroform, chloroform-methanol, and finally with methanol. The various fractions were recombined according to their paper chromatographic behavior. Crystallization from methanol of the alkaloids recovered from the benzene, benzene-chloroform, and chloroform eluates gave *l*-curine. The chloroform-1% methanol and chloroform-3% methanol eluates gave a mixture of *l*-curine and a second higher melting alkaloid. Fractional crystallization from methanol ultimately gave 812 mg. of *l*-curine and 128 mg. of the second alkaloid, m. p. 318–319° (decompn.),  $[\alpha]_D^{25} +59^\circ$  (c 1.15, pyr.);  $\lambda_{max}^{0.1\% \text{ } N^+HCl}$  273.5  $\mu$  ( $\epsilon$  4,500), 282  $\mu$  ( $\epsilon$  4,400). The melting point was not depressed on admixture with an authentic sample of *d*-isochondrodendrine.<sup>6</sup> The paper chromatographic behavior<sup>6</sup> and infrared spectrum (KBr pellet) were identical with those of the authentic *d*-isochondrodendrine sample. Methylation with diazomethane afforded the known dimethyl ether of *d*-isochondrodendrine, cycleanine, m. p. 273–274°,  $[\alpha]_D^{25} -8.5^\circ$  (c 4.65,  $CHCl_3$ )  $\lambda_{max}^{alc}$  276  $\mu$  ( $\epsilon$  4,000), 284  $\mu$  ( $\epsilon$  3,800). The melting point was not depressed on admixture of an authentic sample of cycleanine.<sup>6</sup> The paper chromatographic behavior and infrared spectrum in chloroform were identical with those of the authentic cycleanine sample.

alumina (20 Gm.) by the procedure described above gave 387 mg. of *l*-curine, 440 mg. of *d*-isochondrodendrine, and 210 mg. of hayatin. The paper chromatographic behavior<sup>6</sup> of the noncrystalline fractions indicated the presence of *l*-curine as a principal component.

*Fraction F*.—Extraction of this highly insoluble fraction in a Soxhlet extractor with methanol for two days followed by concentration of the methanol extract yielded 2.46 Gm. of *l*-curine. Further extraction with pyridine failed to give any additional alkaloidal material.

## PHARMACOLOGICAL RESULTS

Preliminary pharmacological tests were performed on the total methanol-extractable alkaloids (fractions A, B, and C), on the methiodide prepared therefrom, and on the quaternary alkaloid fraction. The nonquaternary bases produced only bradypnea and moderate decreased activity after doses of 2,000  $\mu$ g./Kg. orally in the mouse. Intraperitoneally these alkaloids produced prolonged depression of motor activity after doses as low as 50 or 100  $\mu$ g./Kg., and lethal effects at 250  $\mu$ g./Kg. This material differed slightly from the methiodide or *d*-tubocurarine. With the latter two, no overt effects or transient CNS depressing effects are observed below lethal doses. With the nonquaternary bases parenteral doses below the lethal dose caused depression for more than eight hours. After an acute intravenous dose of 2.5 to 10.0  $\mu$ g./Kg. in the pentobarbitalized cat, the nonquaternary bases produced respiratory arrest. Hypotension was accompanied by respiratory depression at nontoxic dose levels. Only the responses to peripheral vagus stimulation were blocked or diminished. No other significant autonomic changes were observed.

The methiodide mixture produced exophthalmia, depression of motor activity, dyspnea, asphyxia,

mg./Kg (cumulative 4.0 mg./Kg.). Larger doses of 5 or 10 mg./Kg. proved lethal due to respiratory arrest. No autonomic effects were noted. None of the alkaloid fractions showed significant diuretic activity in the rat.

## REFERENCES

- (1) Chopra, R N., Chopra, I. C., Handa, K. L., and Kapur, L. D., "Indigenous Drugs of India," 2nd Ed., V N Dhar and Sons, Calcutta, India, 1958.
- (2) Wiggers, A., *Ann*, 33, 81 (1810).
- (3) Scholtz, M., *Ber*, 29, 2054 (1896).
- (4) Scholtz, M., *Arch Pharm*, 237, 199 (1899).
- (5) Bhattacharji, S., Sharma, V. N., and Dhar, M. L., *J Sci Ind Res*, India, 15B, 363 (1956).
- (6) Pradhan, S. N., and De, N. N., *Brit J Pharmacol*, 8, 399 (1953).
- (7) Piso, *De Facultatibus simplicium*, 4, 94 (1648).
- (8) Margraaf, *Hist Plantarum*, 1, 25 (1648).
- (9) Linné, C. V., "Species Plantarum," Impensis Direct Laurentii Salvii, Holmicæ, 1763, p. 1473.
- (10) Woodville, W., "Medical Botany," James Phillips and George Yard, London, England, 1790, p. 227.
- (11) Loudon, J. C., "An Encyclopedia of Plants," Longman, Orme, Brown, Green and Longman, London, England, 1841, pp. 848-850.
- (12) "United States Pharmacopoeia," Gregg and Elliot, Philadelphia, Pa., 1842, p. 47.
- (13) *Ibid*, Lippincott, Groomb and Co., Philadelphia, Pa., 1851, p. 53.

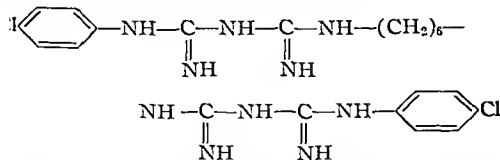
- (14) *Ibid*, 4th rev, J B Lippincott and Co, Philadelphia, Pa, 1864, p 41
- (15) *Ibid*, 5th rev, J B Lippincott and Co, Philadelphia, Pa, (1873), p 41.
- (16) Hanbury, D, *Pharm J*, 33, 81, 102(1873)
- (17) "United States Pharmacopoeia," 6th rev, William Wood and Co, New York, N Y, 1882, p 247
- (18) "The National Formulary," 4th ed, American Pharmaceutical Association, Washington, D C, 1916, p 326
- (19) *Ibid*, 5th ed, 1926, p 372
- (20) Flückiger, F A, and Hanbury, D, "Pharmacographia—A History of the Principal Drugs of Vegetable Origin," 2nd Ed, MacMillan and Co, London, England, 1879, pp 25-31
- (21) Bentley, R, and Trimen, H, "Medicinal Plants," Vol I, J and A Churchill, London, England, 1880, p 15
- (22) Engler, A, and Prantl, K, "Die Natürlichen Pflanzenfamilien," III Teil, 2 Abteilung, Verlag von Wilhelm Engelmann, Leipzig, Germany, 1891, p 84
- (23) Millspaugh, C F, "Medicinal Plants," Vol I, John C Yorston and Co, Philadelphia Pa, 1892, p 14-22
- (24) Tschreul, A, "Handbuch der Pharmakognosie," Band III, Abteilung I, Verlag von Cbr Herm Tauchnitz, Leipzig, Germany, 1923, p 730
- (25) Engler, A, "Syllabus der Pflanzenfamilien," Verlag von Gebrüder Borntraeger, Berlin, Germany, 1924, p 207
- (26) Krukoff, B A, and Moldenke, H N., *Brittonia*, 3, 1(1938)
- (27) Scholtz, M and Koch, O, *Arch Pharm*, 252, 513(1914)
- (28) Faltis, F and Neumann, F, *Monatsh*, 42, 311(1921)
- (29) King, H, *J Chem Soc*, 1940, 737
- (30) Tomita, M, and Kikuchi, T, *J Pharm Soc Japan*, 77, 69(1957)
- (31) Tomita, M, and Watanabe, Y, *ibid*, 76, 686(1956)

## Antimicrobial Activity, *In Vitro*, of Chlorhexidine<sup>\*</sup>

By C. A. LAWRENCE

Comparative *in vitro* tests indicate that chlorhexidine has greater antibacterial activity than benzalkonium chloride, a combination of *o*-phenyl- and *p*-*tert*-amyl-phenol, and an iodine-polyvinylpyrrolidone complex under the test conditions. Chlorhexidine in relatively high dilutions was effective against several cultures of Gram-negative bacteria in the phenol coefficient test. In moderate concentrations, it was active against staphylococci and anaerobes.

AN INCREASING number of reports in the British and Canadian literature describe the strong antimicrobial activity and clinical properties of a new germicide chlorhexidine, bis(*p*-chlorophenyl-diguanido)hexane,<sup>1</sup> which has the following chemical configuration



\* Received December 18, 1959, from the Los Angeles County Health Department and the Department of Infectious Diseases, University of California Medical Center, Los Angeles.

<sup>1</sup> Hibitane, Imperial Chemical (Pharmaceuticals) Ltd., England, available in the United States from Ayerst Laboratories, Inc., New York, N. Y.

The compound is a colorless, odorless, strongly basic, highly insoluble salt with a melting point of 134°. The diacetate substance has a melting point of 154° and is soluble to approximately 1.9 per cent in water, whereas the dihydrochloride has a melting point of 275° and has a solubility of only 0.06 per cent (1).

As with other germicides, chlorhexidine is reduced somewhat in antimicrobial activity by certain organic substances, i. e., milk, blood, pus, etc.; however, this reduction is less marked than that observed with some of the other commonly used germicides (2). In mouse injection experiments, the compound still exerts its full effect when suspended in 0.2 per cent soap, is compatible with nonionic and cationic detergents (1).



The paper chromatographic behavior<sup>5</sup> and infrared spectrum in chloroform solution were identical with those of the authentic *l*-curine sample.

**Fraction B**—Crystallization of the crude alkaloid fraction (610 mg.) from methanol gave 420 mg. of *l*-curine.

**Fraction C**.—Crystallization of the crude alkaloid fraction (50 Gm.) from methanol afforded 41.5 Gm. of a crude microcrystalline mixture, m. p. 209–211°. A portion of the microcrystalline mixture (1.105 Gm.) was chromatographed on Woelm "neutral" alumina (20 Gm.). The alkaloid was applied to the column as a solution in benzene and the column was developed by successive elution with benzene-chloroform, chloroform, chloroform-methanol, and finally with methanol. The various fractions were recombined according to their paper chromatographic behavior. Crystallization from methanol of the alkaloids recovered from the benzene, benzene-chloroform, and chloroform eluates gave *l*-curine. The chloroform-1% methanol and chloroform-3% methanol eluates gave a mixture of *l*-curine and a second higher melting alkaloid. Fractional crystallization from methanol ultimately gave 812 mg of *l*-curine and 128 mg. of the second alkaloid, m. p. 318–319° (decompn.),  $[\alpha]_D^{25} +59^\circ$  (c 1.15, pyr.);  $\lambda_{\max}^{0.1\% \text{ } N \text{ HCl}}$  273.5 m $\mu$  ( $\epsilon$  4,500), 282 m $\mu$  ( $\epsilon$  4,400). The melting point was not depressed on admixture with an authentic sample of *d*-isochondrodendrine.<sup>6</sup> The paper chromatographic behavior<sup>5</sup> and infrared spectrum (KBr pellet) were identical with those of the authentic *d*-isochondrodendrine sample. Methylation with diazomethane afforded the known dimethyl ether of *d*-isochondrodendrine, cycleanine, m. p. 273–274°,  $[\alpha]_D^{25} -8.5^\circ$  (c 4.65, CHCl<sub>3</sub>)  $\lambda_{\max}^{alc}$  276 m $\mu$  ( $\epsilon$  4,000), 284 m $\mu$  ( $\epsilon$  3,800). The melting point was not depressed on admixture of an authentic sample of cycleanine.<sup>6</sup> The paper chromatographic behavior and infrared spectrum in chloroform were identical with those of the authentic cycleanine sample.

**Fraction D**—Chromatography of the alkaloid fraction by the procedure described above yielded 2.70 Gm. of *l*-curine, 110 mg. of *d*-isochondrodendrine, and 105 mg. of a third, high melting compound. Recrystallization of the third compound from aqueous pyridine gave colorless microcrystals, m. p. 301–302° (decompn.);  $[\alpha]_D^{25} 0^\circ$  (c 1.02, pyr.);  $\lambda_{\max}^{0.1\% \text{ } N \text{ HCl}}$  282 m $\mu$  ( $\epsilon$  8,200). The melting point was not depressed on admixture with an authentic sample of hayatin.<sup>7</sup> The paper chromatographic behavior<sup>5</sup> and infrared spectrum (KBr pellet) were identical with those of the authentic hayatin sample.

**Fraction E**.—Crystallization of the alkaloid fraction (7 Gm.) from methanol gave 2.8 Gm. of a crude microcrystalline mixture, m. p. 260–265°. Chromatography of a portion (1.10 Gm.) on neutral

alumina (20 Gm.) by the procedure described above gave 387 mg. of *l*-curine, 440 mg. of *d*-isochondrodendrine, and 210 mg. of hayatin. The paper chromatographic behavior<sup>5</sup> of the noncrystalline fractions indicated the presence of *l*-curine as a principal component.

**Fraction F**.—Extraction of this highly insoluble fraction in a Soxhlet extractor with methanol for two days followed by concentration of the methanol extract yielded 2.46 Gm. of *l*-curine. Further extraction with pyridine failed to give any additional alkaloidal material.

## PHARMACOLOGICAL RESULTS

Preliminary pharmacological tests were performed on the total methanol-extractable alkaloids (fractions A, B, and C), on the methiodide prepared therefrom, and on the quaternary alkaloid fraction.<sup>2</sup> The nonquaternary bases produced only bradypnea and moderate decreased activity after doses of 2,000 mg./Kg. orally in the mouse. Intraperitoneally, these alkaloids produced prolonged depression of motor activity after doses as low as 50 or 100 mg./Kg., and lethal effects at 250 mg./Kg. This material differed slightly from the methiodide or *d*-tubocurarine. With the latter two, no overt effects or transient CNS depressing effects are observed below lethal doses. With the nonquaternary bases, parenteral doses below the lethal dose caused depression for more than eight hours. After an acute intravenous dose of 2.5 to 10.0 mg./Kg. in the pentobarbitalized cat, the nonquaternary bases produced respiratory arrest. Hypotension was accompanied by respiratory depression at nontoxic dose levels. Only the responses to peripheral vagal stimulation were blocked or diminished. No other significant autonomic changes were observed.

The methiodide mixture produced exophthalmia, depression of motor activity, dyspnea, asphyxial convulsions, and acute death in mice after oral doses of 1,000 mg./Kg. Lower doses failed to produce overt biological activity. Intraperitoneally, this material caused absence of grasp reflex, asphyxial convulsions, and acute death after doses as low as 10 mg./Kg. No overt effects occurred at 1.0 mg./Kg. The methiodide caused hypotension in pentobarbitalized cats, but a very narrow range exists between the effective dose level and the toxic dose level.

The quaternary alkaloid fraction produced slight motor depression in mice after an oral dose of 500 mg./Kg. Larger doses caused marked depression and toxic manifestations with death at the 2,000 mg./Kg. dose level. Observed gross effects in mice: at 250 mg./Kg. p. o., no overt effects; at 500 mg./Kg. p. o., slight depression; at 1,000 mg./Kg. p. o., marked depression, low posture, dyspnea, slight ataxia, intention tremors, diarrhea (1/2); at 2,000 mg./Kg. p. o., dyspnea, marked depression, intention tremors, retching, exophthalmia, cyanosis, asphyxial convulsions, death (eight to twelve minutes). No significant alterations in pain threshold, pupil size, or body temperature were noted at the tested dose levels. In cats anesthetized with chloralose, the quaternary alkaloid fraction produced significant and sustained lowering of mean arterial blood pressure after acute intravenous dosage of 2.5

<sup>5</sup> The procedure and solvent system used were essentially those of Tomita and Watanabe (31). The method involved the use of paper pretreated with buffer at pH 3.5 and the detection of alkaloidal spots with a chloroform solution of bromophenol blue. The solvent system was the upper layer of a mixture of *n*-butanol:acetic acid:water (67:10:23 by volume) prepared by shaking well and allowing to stand at room temperature for two days.

<sup>6</sup> The authors thank Professor Masao Tomita, Kyoto University, Japan, for authentic samples of *d*-isochondrodendrine and its O,O-dimethyl ether, cycleanine.

<sup>7</sup> The authors thank Dr. S. Bhattacharji, Central Drug Research Institute, Lucknow, India, for an authentic sample of bayatin.

mg/Kg (cumulative 40 mg./Kg.). Larger doses of 5 or 10 mg/Kg proved lethal due to respiratory arrest. No autonomic effects were noted. None of the alkaloid fractions showed significant diuretic activity in the rat

REFERENCES

(1) Chopra, R. N., Chopra, I. C., Hnuda, K. L. and Kapur, L. D., "Indigenous Drugs of India," 2nd Ed., V. N. Dhar and Sons, Calcutta, India, 1958  
(2) Wiggers, A., *Ann*, 33, 81(1840)  
(3) Scholtz, M., *Ber*, 29, 2054(1896)  
(4) Scholtz, M., *Arch Pharm*, 237, 199(1899)  
(5) Bhattachary, S., Sharma, V. N., and Dhar, M. L., *J Sci Ind Research India*, 15B, 363(1956)  
(6) Pradhan, S. N., and De N. N., *Brit J Pharmacol*, 8, 399(1953)  
(7) Piso, *De Facultatibus simplicium*, 4, 94(1648)  
(8) Marcgrav, *Hist Plantarum*, 1, 25(1648)  
(9) Linné, C. V., "Species Plantarum," Impensis Direct Laurentii Salvii, Holmiae, 1763, p. 1473  
(10) Woodville, W., "Medical Botany," James Phillips and George Yard, London, England, 1790, p. 227  
(11) Loudon, J. C., "An Encyclopedia of Plants," Longman, Orme, Brown, Green and Longman, London, England, 1841, pp. 848-850  
(12) "United States Pharmacopeia," Gregg and Eliot, Philadelphia, Pa., 1842, p. 47  
(13) *Ibid*, Lippincott, Grombo and Co., Philadelphia, Pa., 1851, p. 53

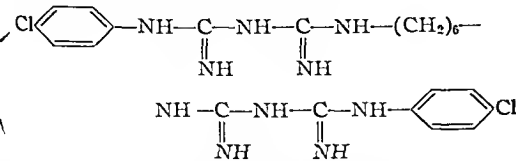
(14) *Ibid*, 4th rev., J. B. Lippincott and Co., Philadelphia, Pa., 1864, p. 41  
(15) *Ibid*, 5th rev., J. B. Lippincott and Co., Philadelphia, Pa., (1873), p. 41.  
(16) Hanbury, D., *Pharm J*, 33, 81, 102(1873)  
(17) "United States Pharmacopeia," 6th rev., William Wood and Co., New York, N. Y., 1882, p. 247  
(18) "The National Formulary," 4th ed., American Pharmaceutical Association, Washington, D. C., 1916, p. 326  
(19) *Ibid*, 5th ed., 1926, p. 372  
(20) Fluckiger, F. A., and Hanbury, D., "Pharmacographia—A History of the Principal Drugs of Vegetable Origin," 2nd Ed., MacMillan and Co., London, England, 1879, pp. 25-31  
(21) Bentley, R., and Trimen, H., "Medicinal Plants," Vol. 1, J. and A. Churchill, London, England, 1880, p. 15  
(22) Engler, A., and Prantl, K., "Die Natürlichen Pflanzenfamilien" 111 Teil, 2 Abteilung, Verlag von Wilhelm Engelmann, Leipzig Germany 1891, p. 84  
(23) Millsbaugh C. F., "Medicinal Plants," Vol. I, John C. Yorston and Co., Philadelphia, Pa., 1892, p. 14-22  
(24) Tschirch A., "Handbuch der Pharmakognosie," Band 111, Abteilung 1 Verlag von Cbr Herm Tauchnitz, Leipzig, Germany, 1923, p. 730  
(25) Engler, A., "Syllabus der Pflanzenfamilien," Verlag von Gebrüder Borntraeger, Berlin, Germany, 1924, p. 207  
(26) Krukov, B. A., and Moldenke, H. N., *Brittonia*, 3, 1(1938)  
(27) Scholtz, M. and Koebe, O., *Arch Pharm*, 252, 513(1914)  
(28) Faltis F. and Neumann, F. *Monatsh*, 42, 311(1921)  
(29) King, H., *J Chem Soc*, 1940, 737  
(30) Tomita, M., and Kikuchi, T., *J Pharm Soc Japan*, 77, 69(1957)  
(31) Tomita, M., and Watanabe, Y., *ibid*, 76, 686(1956)

Antimicrobial Activity, *In Vitro*,  
of Chlorhexidine\*

By C. A. LAWRENCE

Comparative *in vitro* tests indicate that chlorhexidine has greater antibacterial activity than benzalkonium chloride, a combination of *o*-phenyl- and *p*-*tert*-amylphenol, and an iodine-polyvinylpyrrolidone complex under the test conditions. Chlorhexidine in relatively high dilutions was effective against several cultures of Gram-negative bacteria in the phenol coefficient test. In moderate concentrations, it was active against staphylococci and anaerobes.

AN INCREASING number of reports in the British and Canadian literature describe the strong antimicrobial activity and clinical properties of a new germicide chlorhexidine, bis(*p*-chlorophenyl-diguanido)hexane,<sup>1</sup> which has the following chemical configuration



\* Received December 18, 1959, from the Los Angeles County Health Department and the Department of Infectious Diseases, University of California Medical Center, Los Angeles  
<sup>1</sup> Hibitane, Imperial Chemical (Pharmaceuticals) Ltd., England, available in the United States from Ayerst Laboratories, Inc., New York, N. Y.

The compound is a colorless, odorless, strongly basic, highly insoluble salt with a melting point of 134°. The diacetate substance has a melting point of 154° and is soluble to approximately 1.9 per cent in water, whereas the dihydrochloride has a melting point of 275° and has a solubility of only 0.06 per cent (1).

As with other germicides, chlorhexidine is reduced somewhat in antimicrobial activity by certain organic substances, i. e., milk, blood, pus, etc.; however, this reduction is less marked than that observed with some of the other commonly used germicides (2). In mouse injection experiments, the compound still exerts its full effect when suspended in 0.2 per cent soap, is compatible with nonionic and cationic detergents (1),

TABLE I—LIMITING DILUTIONS OF ANTIBACTERIAL AGENTS SHOWING BACTERIOSTATIC (BS) AND BACTERICIDAL (BC) ACTIVITIES AGAINST VARIOUS BACTERIA IN NUTRIENT MEDIA

Organisms	Disinfectant Limiting Dilution Bacteriostatic (BS) and Bactericidal (BC) Agent I <sup>a</sup> Agent II <sup>b</sup> Agent III <sup>c</sup> Agent IV <sup>d</sup>							
	BS	BC	BS	BC	BS	BC	BS	BC
<i>Salmonella typhosa</i>	600,000	600,000	80,000	80,000	1,000	1,000	4,000 <sup>e</sup>	2,000
<i>Pseudomonas aeruginosa</i>	100,000	100,000	10,000	10,000	100	80	4,000	4,000
<i>Proteus vulgaris</i>	80,000	80,000	40,000	40,000	2,000	2,000	4,000	4,000
<i>Escherichia coli</i>	800,000	800,000	60,000	60,000	1,000	1,000	2,000	2,000
<i>Staphylococcus aureus</i> No 209	1,000,000	1,000,000	800,000	600,000	6,000	6,000	6,000	6,000
<i>Staphylococcus aureus</i> No 261 <sup>f</sup>	1,000,000	1,000,000	1,000,000	1,000,000	4,000	4,000	10,000	10,000
<i>Bacillus cereus</i>	400,000	80,000	200,000	200,000	10,000	10,000	6,000	4,000
<i>Bacillus subtilis</i>	1,000,000	1,000,000	800,000	800,000	8,000	8,000	4,000	4,000
<i>Clostridium novyi</i>	800,000	100,000	100,000	60,000	20,000	4,000	10,000	<1,000 <sup>e</sup>
<i>Clostridium tetani</i>	200,000	200,000	200,000	200,000	10,000	10,000	6,000	6,000
<i>Clostridium botulinum</i>	400,000	200,000	200,000	200,000	10,000	10,000	6,000	6,000
<i>Clostridium perfringens</i>	100,000	100,000	200,000	200,000	6,000	6,000	2,000	2,000

<sup>a</sup> Chlorhexidine <sup>b</sup> Benzalkonium chloride <sup>c</sup> A phenolic type disinfectant <sup>d</sup> An iodine polyvinylpyrrolidone complex  
<sup>e</sup> Dilutions based upon iodine content of iodine PVP complex <sup>f</sup> An antibiotic resistant strain of phage type 52, 42B, 44A, 81  
<sup>g</sup> Highest concentration tested failed to show bactericidal activity

TABLE II—LIMITING DILUTIONS OF ANTIBACTERIAL AGENTS SHOWING FUNGISTATIC (FS) AND FUNGICIDAL (FC) ACTIVITIES AGAINST FUNGI IN NUTRIENT MEDIUM

Organisms	Disinfectant Limiting Dilution Fungistatic (FS) and Fungicidal (FC) Agent I <sup>a</sup> Agent II <sup>b</sup> Agent III <sup>c</sup> Agent IV <sup>d</sup>							
	FS	FC	FS	FC	FS	FC	FS	FC
<i>Microsporum audouinii</i>	100,000	100,000	100,000	100,000	60,000	40,000	10,000 <sup>e</sup>	10,000
<i>Microsporum gypseum</i>	100,000	100,000	80,000	80,000	10,000	10,000	4,000 <sup>e</sup>	4,000
<i>Microsporum canis</i>	80,000	80,000	100,000	100,000	40,000	40,000	8,000	8,000
<i>Trichophyton tonsurans</i>	100,000	100,000	100,000	100,000	10,000	10,000	8,000	8,000
<i>Trichophyton mentagrophytes</i>	600,000	600,000	400,000	400,000	60,000	40,000	40,000	40,000

<sup>a</sup> <sup>b</sup> <sup>c</sup> and <sup>d</sup> See footnotes Table I <sup>e</sup> Dilutions based upon iodine content of iodine PVP complex

and has been used successfully in creams with penicillin (3, 4), neomycin, and bacitracin (5). In effective germicidal concentrations the compound has been reported to have little or no untoward effects in acute and chronic toxicity studies in animals (1), and is well tolerated when applied topically to the skin of humans (2, 5-8). Chlorhexidine has been used successfully in reducing the incidence of postoperative infections by the treatment of instruments (2, 9, 10), disinfection of urine bottles (11), bedpans (12), and rubber gloves (13). It has also been suggested for use as a spray to prevent the spread of dust-borne infections in hospitals (14).

The compound, incorporated in a cream base, is being used extensively in the British Commonwealth to control staphylococcal infections in hospitals. A hand cream containing the germi-

cide was found effective when used by physicians and nurses to reduce the incidence of infections in maternity wards and nurseries (15-20). A cream has also been applied effectively to the skin of infants in the prevention of staphylococcal infections (21). In combination with certain antibiotics, chlorhexidine in a cream base, when applied to the nasal cavities of staphylococcal carriers, has been considered an effective means for controlling the incidence of infections in hospital wards and also in persons outside of hospitals who were found to have recurrences of minor staphylococcal infections from nasal colonies of *Staphylococcus aureus* (5, 22).

Aqueous and alcoholic solutions of chlorhexidine have also been used successfully in burns (6), in dermatology for the treatment of fungal diseases (21, 23), in obstetrics and gynecology

(2, 21), as a skin antiseptic for preoperative preparation of the patient's skin, and for the surgeon's hand rinse (7, 8, 24, 25)

At the present writing, there have been but few references to the suggested use of chlorhexidine as a germicide published in the American literature (26). It is for this reason that a study was undertaken to determine the comparative antimicrobial properties, *in vitro*, of chlorhexidine with those of three other classes of disinfectants in common use in this country. The latter agents included a quaternary ammonium germicide, benzalkonium chloride,<sup>2</sup> a phenolic type disinfectant,<sup>3</sup> and an iodine-polyvinylpyrrolidone complex.<sup>4</sup>

## EXPERIMENTAL

**Bacteriostatic and Bactericidal Tests.**—Primary dilutions of the disinfectants were made in distilled water and 1 ml of the appropriate dilution added to 9 ml of sterile beef extract broth in the studies in which the aerobic bacteria were tested. Brewer's fluid thioglycollate medium was used for the anaerobes (*Clostridia*). Each tube was inoculated with a loopful of a twenty-four hour broth culture of test organism. The tubes were incubated at 37° for seventy two hours and the presence or absence of visible growth recorded. Tubes showing no growth at this time were checked for bactericidal activity by transferring three loopfuls of the test mixture to tubes of sterile medium without added disinfectant. The latter tubes were incubated for an additional seventy two hours. Failure of growth to occur in the subculture tubes was taken as evidence that the organisms had not survived or had been killed in the original broth disinfectant tubes. The results of this study are presented in Table I.

**Results**—From the data given in Table I it will be noted that of the four disinfectants tested, chlorhexidine in general gives an overall antibacterial activity greater than the other three germicides tested. In some instances the bacteria were affected by comparable concentrations of benzalkonium chloride and chlorhexidine. This was particularly true with the antibiotic resistant strain of *Staphylococcus aureus* (No. 261) and some of the anaerobes.

Under the same testing conditions, the phenolic disinfectant was found to be far less active than chlorhexidine and benzalkonium chloride against all of the organisms examined in the presence of the nutrient (proteinaceous) media. In general, the iodine containing germicide showed an activity comparable with that of the phenolic preparation.

**Fungistatic and Fungicidal Tests**—One milliliter of the distilled water dilutions of the disinfectants was added to 9 ml of Neopeptone medium consisting of glucose, 4 Gm., Bacto Neopeptone, 1 Gm. distilled water 100 ml., pH, 6.0. The fungi were first grown for a period of one month at room temperature in four ounce screw capped vials containing

glass beads and the medium described above. At the end of the incubation period, the heavy mycelial and spore mass was shaken vigorously with the glass beads and the suspension filtered through several layers of sterile gauze. A loopful of this suspension was added to each tube of broth disinfectant solution.

The tubes were incubated for one month at room temperature and those showing no growth at this time were checked for fungistatic activity by subculturing three loopfuls to tubes of medium without disinfectant. The latter were again incubated for one month and the presence or absence of visible growth recorded. The results of this study are presented in Table II.

**Results**—Examination of the data given in Table II will reveal a close correlation in fungistatic and fungicidal activities between chlorhexidine and benzalkonium chloride, similar to the comparable effects of these two germicides against bacteria (Table I). The phenolic preparation is somewhat lower in antifungal properties than the latter two agents but shows a greater action against these organisms than against bacteria. In descending order of activity, an iodine polyvinylpyrrolidone complex will be noted to have the least activity against the fungi studied.

**Phenol Coefficient Test**—The A O A C (27) method for determining phenol coefficients was used in determining the germicidal activity of chlorhexidine. The tubes containing the dilutions of the latter agent and tubes of phenol were maintained in a water bath at 20° during the time intervals of transfer. In all instances the inoculum consisted of twenty four hour cultures of the respective organisms grown at 37°. The results of this study are presented in Table III.

**Results**—In summarizing the data given in Table III it will be noted that chlorhexidine, in general,

TABLE III—PHENOL COEFFICIENT (A O A C METHOD) OF CHLORHEXIDINE AGAINST A VARIETY OF BACTERIA<sup>1</sup>

Organisms	Limiting Dilutions Effective in Ten Minutes at 20°		Phenol Coefficient
	Chlorhexidine	Phenol	
<i>Salmonella typhosa</i>	1:40,000	1:120	333
<i>Pseudomonas aeruginosa</i>	1:15,000	1:100	150
<i>Proteus vulgaris</i>	1:5,000	1:100	50
<i>Escherichia coli</i>	1:25,000	1:100	250
<i>Staphylococcus aureus</i> No. 209	1:10,000	1:80	125
<i>Staphylococcus aureus</i> No. 261 <sup>1</sup>	1:7,500	1:70	107
<i>Bacillus cereus</i>	1:70,000	1:130	538
<i>Bacillus subtilis</i>	1:5,000	1:50	100
<i>Clostridium novyi</i>	1:5,000	1:20	250
<i>Clostridium tetani</i>	1:5,000	1:140	36
<i>Clostridium botulinum</i>	1:2,500	1:190	13
<i>Clostridium perfringens</i>	1:2,500	1:130	19

<sup>1</sup> All of the cultures used in this test were incubated at 37° for twenty four hours prior to testing. The *Clostridia* were cultured and transferred in fluid thioglycollate medium. All of the remaining organisms were grown in beef extract broth.

<sup>2</sup> See footnote 1, Table I.

<sup>2</sup> Zephiran chloride. Winthrop Stearns Inc. New York, N. Y.

<sup>3</sup> Amphyl (o phenylphenol and p tert amylphenol). Ichn and Fink Products Corp. New York, N. Y.

<sup>4</sup> Iodine. Iodine Pharmaceutical Corp. New York, N. Y.

shows a high germicidal activity against the Gram-negative bacteria, with dilutions ranging from 1 15,000 (*Pseudomonas aeruginosa*) to 1 40,000 (*Salmonella typhosa*) effective in ten minutes. The one exception in this group of bacteria was *Proteus vulgaris* which required a 1 5,000 dilution to effect complete destruction of this organism. The effective germicidal range of chlorhexidine against the two strains of *S. aureus* was between 1 7,500 to 1 10,000.

While the strain of *Bacillus cereus* used in this study appeared to be relatively resistant to chlorhexidine in the broth dilution test (Table I), the culture was highly sensitive to the disinfectant when tested in an aqueous dilution of the germicide. As noted in Table III, a dilution of 1 70,000 was effective against this organism. Also, a dilution of 1 130 phenol proved to be germicidal for this organism. *Bacillus subtilis*, on the other hand was considerably more resistant to both germicides, since concentrations of 1 5,000 and 1 50 of chlorhexidine and phenol, respectively, were required to destroy this organism.

The twenty-four-hour cultures of the anaerobes required concentrations of 1 2,500 to 1 5,000 of chlorhexidine for complete destruction. With the exception of the strain of *Clostridium novyi*, which required a concentration of 1 50 phenol for effective kill, the remaining clostridia failed to grow after being exposed to dilutions ranging from 1 130 to 1 190 of the latter germicide.

## SUMMARY AND CONCLUSIONS

The present report compares the antimicrobial activity, *in vitro*, of chlorhexidine with that of a quaternary ammonium disinfectant, a phenolic compound, and an iodophor. In general, the new compound showed greater antibacterial activity than the other classes of disinfectants when tested in the presence of nutrient media. It was also found to be highly effective against several strains of dermatophytes.

Using the phenol coefficient test, chlorhexidine, in relatively high dilutions, was found to be effective against several cultures of Gram-negative bacteria. In moderate concentrations, the compound was found to be active against staphylococci and anaerobes.

Reference is made to a number of British publications in which chlorhexidine has been reported to be an effective adjunct in reducing the incidence of postoperative infections and the control of staphylococcal infections in hospitals.

## REFERENCES

- (1) Ayerst Laboratories Research Council Report, 1959
- (2) Calman, R. M., and Murray, J., *Brit Med J*, 2, 200 (1956)
- (3) Lowbury, E. J. L., *ibid*, 1, 985 (1955)
- (4) Lowbury, E. J. L., *Lancet*, 2, 305 (1957)
- (5) Gould, J. C., and Cruikshank, J. D., *ibid*, 2, 1157 (1957)
- (6) Grant, J. C., and Findlay, J. C., *ibid*, 1, 862 (1957)
- (7) Myers, G. E., MacKenzie, W. C., and Ward, K. A., *Can J Microbiol*, 2, 87 (1956)
- (8) ".....", 2, 1164 (1958)
- (9) ".....", W. A. Linton, K. B. Slade, N., and ".....", 608 (1958)
- (10) Beeuwkes, H., and de Vries, H. R., *ibid*, 2, 913 (1956)
- (11) Stephens, N. A., and Henriques, C. Q., *ibid*, 2, 357 (1956)
- (12) McLeod, J. W., *ibid*, 1, 394 (1958)
- (13) Calnan, J., *Brit Med J*, 1, 1184 (1957)
- (14) Marsh, F., *Lancet*, 2, 1196 (1955)
- (15) Monro, J. A., and Markham, N. P., *ibid*, 2, 186 (1958)
- (16) Murray, J., and Calman, R. M., *Brit Med J*, 1, 81 (1955)
- (17) Gillespie, W. A., and Alder, V. C., *Lancet*, 1, 632 (1957)
- (18) Gillespie, W. A., Simpson, K., and Tozer, R. C., *ibid*, 2, 1075 (1958)
- (19) Speirs, A. L., *Nursing Mirror*, 106, vii (1957)
- (20) Jellard, J., *Brit Med J*, 1, 925 (1957)
- (21) Beeuwkes, H., *J Microbiol Serol*, 24, 49 (1958)
- (22) Lowbury, E. J. L., *Lancet*, 1, 513 (1958)
- (23) Van der Meiren, L., Moriamé, G., Achten, G., and Ledoux-Corbusier, M., *Arch belges dermat et syph*, 14, 14 (1958)
- (24) Editorial, *Brit Med J*, 1, 534 (1958)
- (25) Editorial, *ibid*, 1, 1191 (1958)
- (26) "Suggestions for Control of Staphylococcal Infections in Newborn Nurseries," American Academy of Pediatrics, March 1958
- (27) "Official and Tentative Methods of Analysis," 7th ed., Association of Official Agricultural Chemists, Washington, D. C., 1950, p. 88

## Notes

### Note on Saponins and Their Sapogenins from Strawberry Clover\*

BY E. D. WALTER

A crystalline mixture of saponins was isolated from strawberry clover (*Trifolium fragiferum*). Upon hydrolysis with dilute acid this product yielded the soyasapogenols B and C, a very small quantity of unidentified sapogenin, and several sugars.

SOYASAPOGENOLS B and C and possibly A have previously been isolated from the saponins of alfalfa and ladino clover (1) suggesting a rather frequent occurrence of these sapogenols in legume forages.

#### EXPERIMENTAL

Fresh strawberry clover (45 Kg.) was placed in a stainless steel tank and covered with 95% alcohol (50 gallons) and allowed to stand for five days. A 2-gallon portion was drained, filtered, and concentrated to about one-fourth volume when solids appeared. The material was transferred to a separatory funnel and shaken gently with about one-third volume of ether. The saponin separated in the aqueous layer as minute crystals. This layer was centrifuged and the residue was washed with water, and finally with acetone. The yield of dry saponin was about 1 Gm. or about 0.3% of the dry weight of clover.

With another 2-gallon portion of the extract the saponin was isolated the same way except that the extract was treated with about 40 Gm. of charcoal per gallon and filtered through filter aid. This method required less washing with acetone to remove green material, but the yield was about the same. Both methods were employed to obtain the saponin stock for subsequent studies.

The dry saponin was practically insoluble in water, but appreciably soluble in 50-60% ethanol. When concentrated sulfuric acid was added to the dry material a red color formed almost instantly. This rapid color reaction was found particularly characteristic of the unidentified sapogenin melting at 321°.

No action resulted from contact of this saponin with an isolated strip of rabbit ileum, possibly because of its insolubility in the Tyrode's solution in which the strip was suspended. This is in contrast with the results from alfalfa and ladino saponin (1) and from bur clover saponin (hederin) (2).

Isolation of Sapogenins.—The saponin (0.77 Gm.) was dissolved in 60% ethanol and sulfuric acid was added to make the solution approximately 1 N. The total volume was about 108 ml. This solution was refluxed for sixty hours. Water was added to precipitate the sapogenins and they were then extracted with ether in a separatory funnel. The

ether solution was washed with water to remove sulfuric acid, dehydrated with anhydrous sodium sulfate, and clarified with charcoal. The ether was evaporated and the sapogenins were crystallized from methanol; yield 0.344 Gm. About 45% of the saponin was sapogenin. This method was used to obtain larger quantities of sapogenin.

The acid was removed from the hydrolysate as barium sulfate, and the filtrate from this was used for chromatographic identification of the sugars. The following sugars were detected: glucose, galactose, arabinose, xylose, rhamnose, and glucuronic acid, with xylose predominating. The large number of sugars present suggested that more than one saponin was involved.

The sapogenins were first separated into chloroform-soluble and insoluble fractions. Roughly 90% of the material was soluble in chloroform, while the remainder was largely the high melting (321°) fraction, which gave the rapid color reaction with sulfuric acid.

The chloroform-soluble portion, after crystallization from methanol, was dissolved in benzene and chromatographed on a column of alumina (deactivated with 10% acetic acid in benzene), and eluted with increasing concentrations of methanol in benzene (0.5, 2.5, and 100% methanol). The eluate from 0.5% methanol contained material melting at 235-240° and corresponding to soyasapogenol C. The 2.5% methanol eluate contained a fraction melting at 255-260°, corresponding to soyasapogenol B. The 100% methanol fraction contained a trace of the material melting at 321°.

*Soyasapogenol B*.—The fraction melting at 255-260° was recrystallized from methanol-chloroform yielding material of m. p. 260°,  $[\alpha]_D^{25} + 90.7^\circ$ .

*Anal.*—Calcd. for  $C_{26}H_{40}O_6$ : C, 78.55; H, 10.99. Found: C, 78.80; H, 10.6.

Acetylation of this material and crystallization from methanol-chloroform furnished the triacetate m. p. 180°,  $[\alpha]_D^{25} + 78^\circ$ .

*Anal.*—Calcd. for  $C_{36}H_{56}O_9$ : C, 73.93; H, 9.65. Found: C, 74.0; H, 9.49.

The infrared spectra of this triacetate and that of authentic soyasapogenol B triacetate were identical.

*Soyasapogenol C*.—Recrystallization of the fraction melting at 235-240° in methanol-chloroform yielded a product of m. p. 240°,  $[\alpha]_D^{25} + 66^\circ$ . Meyer, *et al.* (3), reported m. p. 239-240° and  $[\alpha]_D^{25} + 65^\circ$ .

Acetylation of this sapogenol with acetic anhydride and chromatographing on deactivated alumina, followed by elution with petroleum ether-benzene (3:1), and crystallization from methanol furnished

\* Received August 13, 1960, from the Western Regional Research Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, U. S. Dept. of Agriculture, Albany, Calif.

The author is indebted to Arthur Bevenue for paper chromatography of the sugars, to Lawrence White and Geraldine Secor for elemental analyses, and to Edith Gong for the infrared spectra.

the diacetate m p  $204^{\circ}$ ,  $[\alpha]_D^{25} + 59^{\circ}$ . The infrared spectra of this material and that of authentic soyasapogenol C diacetate were identical.

*Anal*—Calcd for  $C_{31}H_{50}O_4$ : C, 77.82; H, 9.99. Found: C, 77.8; H, 9.79.

*Unidentified Sapogenin*—The minor fraction eluted from the alumina column with methanol melted at  $321^{\circ}$ , and had a  $[\alpha]_D^{25} + 97^{\circ}$ . This material was acetylated in pyridine solution with acetic anhydride. The product was put on a deactivated alumina column with Skellysolve benzene (3:1), and was eluted with Skellysolve benzene (1:1), followed by benzene. The benzene eluate, when crystallized from methanol and dried in a vacuum at  $121^{\circ}$ , melted at  $227-228^{\circ}$ .  $[\alpha]_D^{25} - 35.5^{\circ}$ . Infrared spectra of the sapogenin  $\lambda_{max}^{KBr}$  5.87  $\mu$  (and strong hydroxyl bands), and of the acetate  $\lambda_{max}^{KBr}$  5.76, 5.86, and 8.1  $\mu$  indicated the presence

of a carbonyl group. The ultraviolet absorption spectra for the acetate  $\lambda_{max}^{EtOH}$  241.5 m $\mu$ ,  $\epsilon$  23,000, 249 m $\mu$ ,  $\epsilon$  25,900, 258 m $\mu$ ,  $\epsilon$  20,800 indicated a conjugated system. Both compounds gave a yellow color with tetranitromethane, and, in contact with concentrated sulfuric acid, gave a red color instantly.

*Anal*—Calcd for  $C_{36}H_{54}O_7$ : C, 72.2; H, 9.07 for three  $CH_3CO$  groups, 21.5 mol wt, 598.72. Found: C, 72.4; H, 8.72;  $CH_3CO$ , 21.1 mol wt (Rast), 612.

Insufficient quantity of material prevented further characterization of this sapogenin at this time.

#### REFERENCES

- (1) Walter F. D., Bickoff F. M., Thompson C. R., Robinson C. H. and Djerassi C. *J. Am. Chem. Soc.* 77, 4936 (1955).
- (2) Walter F. D. *This Journal* 46, 466 (1957).
- (3) Meyer A., Jeger O. and Ruzicka L. *Helv. Chim. Acta* 33, 672 (1950).

## Note on the Hydrophile-Lipophile Balance of Tragacanth\*

By WALLACE L. GUESS

IN A RECENT publication, Chun, *et al.* (1, 2), reported on their investigation of the HLB of some naturally occurring emulsifying agents. These workers pointed out that while tragacanth should be studied more extensively, they did obtain an HLB value for this product of 13-13.5. This value was obtained by using Span<sup>1</sup> 20 and Span 80 as reference surfactants. It was noted in our laboratories that satisfactory oil in water emulsions could not be produced using the Spans and tragacanth. Therefore, the following work was undertaken.

#### EXPERIMENTAL

The method of Chun, *et al.*, for the determination of the HLB of tragacanth was duplicated as nearly as possible with a modification of the method of shaking the emulsions. In this work, all emulsions were shaken for five minute intervals on a Red Devil paint conditioner at 1,600 oscillations per minute. Every emulsion was shaken for three such periods: (a) once immediately after mixing all components, (b) once at the end of one hour, and (c) finally at the end of twenty-four hours. The emulsions were allowed to stand undisturbed for twenty-four hours and were evaluated by a blind technique (simple

masking of labels) according to degree of creaming and/or oil separation. In order to insure that only oil in water emulsions were being evaluated, a drop dilution test (3) and a water soluble dye test (3) were conducted on each emulsion.

The results of this study are shown in Table I.

#### DISCUSSION

It is interesting to note that in these laboratories no o/w emulsions could be prepared with combinations of Spans and tragacanth, while all the Tweens used in optimum proportions with tragacanth produced smooth, white, slowly creaming o/w emulsions. These observations were true with two different lots of tragacanth. It may also be observed that an average HLB value of 11.92 was determined, which checks fairly well with the value obtained by Chun and his co-workers, particularly since a natural product of such varied physical characteristics as tragacanth was used.

#### SUMMARY

It is recommended that further investigation into the HLB of tragacanth be undertaken to determine if various lots of tragacanth would show a range of HLB values even greater than those referred to in this paper. It may be of interest to determine if tragacanth has an HLB in the true sense as do the synthetic surfactants, or if an HLB value is only an apparent value dependent on viscosity of the tragacanth, per cent insoluble bassorin, and other well known factors.

#### REFERENCES

- (1) Chun A. H. C., Joslin R. S. and Martin A. *Drug & Cosmetic Ind.* 82, 164 (1958).
- (2) Chun A. H. C., Joslin R. S. and Martin A. *ibid.* 82, 112 (1958).
- (3) Martin F. W. *Husa's Pharmaceutical Dispensing*, Mack Publishing Co., Easton Pa. 1959, p. 176.

TABLE I—HLB VALUES OF TRAGACANTH

Tragacanth	—HLB of Tragacanth with—			
	Tween 20	Tween 40	Tween 60	Tween 80
Meyer Bros., lot 163607	11.62	11.90	11.92	11.84
Meer, lot No. 10	12.52	11.90	11.92	11.76
Average HLB	12.07	11.90	11.92	11.80

\* Received May 9, 1960 from the College of Pharmacy, University of Texas at Austin 12.

<sup>1</sup> Trademark Atlas Powder Co., Wilmington, Del.

# Note on Triazinylmethylureas. Guanamines VII<sup>†</sup>

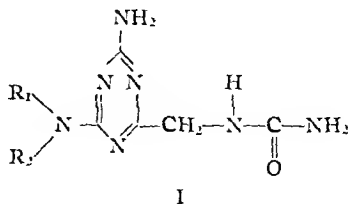
By SEYMOUR L. SHAPIRO, VINCENT A. PARRINO, and LOUIS FREEDMAN

Condensation of ethyl hydantoate with substituted biguanides gave triazinylmethylureas (I) which are pharmacologically active.

MANY urea derivatives have afforded useful pharmacological effects (1), and in this study a series of triazinylmethylureas (I) envisioned as structural analogs of phenacemide (II) (2) have been synthesized. Diphenylhydantoin, interestingly embodies the alkylurea structural features of I, and the acylurea element of II.

The compounds were prepared by condensation (3) of ethyl hydantoate with the appropriate biguanide (4, 5) which afforded the desired I in 5-38% yields of purified product (6).

On pharmacological evaluation (7), compounds 1 and 5 (anticonvulsant), 11 (analgesic), and 3 (anti-inflammatory) gave significant responses



R<sub>1</sub> = alkyl, aralkyl, aryl  
R<sub>2</sub> = H, lower alkyl

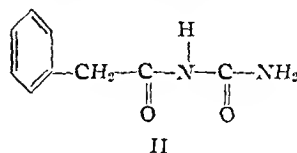


TABLE I—TRIAZINYL METHYLUREAS<sup>a</sup> (SEE FORMULA I)

No.	R <sub>1</sub>	M p., °C <sup>b</sup>	S <sup>c</sup>	Formula	Analyses, % <sup>d</sup>				Nitrogen	
					Carbon Calcd	Carbon Found	Hydrogen Calcd	Hydrogen Found	Calcd	Found
1	C <sub>6</sub> H <sub>5</sub> —	202-203	A	C <sub>8</sub> H <sub>13</sub> N <sub>7</sub> O	43 0	43 1	5 9	5 8		
2	—	231	A	C <sub>10</sub> H <sub>17</sub> N <sub>7</sub> O	47 8	48 1	6 8	7 1		
3 <sup>e</sup>	C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> —	183-184	B	C <sub>13</sub> H <sub>19</sub> N <sub>7</sub> O <sub>2</sub> <sup>h</sup>					32 1	32 7
4	C <sub>6</sub> H <sub>5</sub> CH <sub>2</sub> CH <sub>2</sub> —	204-205	A	C <sub>13</sub> H <sub>17</sub> N <sub>7</sub> O					34 1	33 8
5 <sup>i</sup>	C <sub>6</sub> H <sub>5</sub> —	227	C	C <sub>11</sub> H <sub>13</sub> N <sub>7</sub> O	51 0	51 4	5 1	5 0	37 8	37 6
6 <sup>e</sup>	C <sub>6</sub> H <sub>5</sub> —	238	B	C <sub>12</sub> H <sub>15</sub> N <sub>7</sub> O	52 7	53 2	5 5	5 9	35 9	36 3
7	2—CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> —	209-211	B	C <sub>12</sub> H <sub>15</sub> N <sub>7</sub> O	52 7	52 2	5 5	5 4	35 9	35 5
8 <sup>e</sup>	2—CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> —	247-248	B	C <sub>13</sub> H <sub>17</sub> N <sub>7</sub> O	54 3	54 3	6 0	6 0	34 1	34 4
9 <sup>i</sup>	4—CH <sub>2</sub> C <sub>6</sub> H <sub>4</sub> —	213-214	B	C <sub>14</sub> H <sub>19</sub> N <sub>7</sub> O	55 8	55 4	6 4	6 1		
10	2,6—(CH <sub>2</sub> ) <sub>2</sub> C <sub>6</sub> H <sub>3</sub> —	250	A	C <sub>13</sub> H <sub>17</sub> N <sub>7</sub> O					34 1	34 2
11	2—ClC <sub>6</sub> H <sub>4</sub> —	169	D	C <sub>11</sub> H <sub>12</sub> ClN <sub>7</sub> O					33 4	33 1
12	4—ClC <sub>6</sub> H <sub>4</sub> —	257	A	C <sub>11</sub> H <sub>12</sub> ClN <sub>7</sub> O	45 0	44 9	4 1	3 7	33 4	33 5
13	2—BrC <sub>6</sub> H <sub>4</sub> —	295	A	C <sub>11</sub> H <sub>12</sub> BrN <sub>7</sub> O					29 0	28 5
14	3—BrC <sub>6</sub> H <sub>4</sub> —	218	A	C <sub>11</sub> H <sub>12</sub> BrN <sub>7</sub> O	39 1	39 5	3 6	3 8	29 0	28 6
15	4—CH <sub>2</sub> OC <sub>6</sub> H <sub>4</sub> —	216-217	B	C <sub>12</sub> H <sub>15</sub> N <sub>7</sub> O <sub>2</sub>	49 8	50 1	5 2	5 0		

<sup>a</sup> R<sub>1</sub> = H unless otherwise indicated. <sup>b</sup> Melting points are not corrected and the compounds melted with decomposition. <sup>c</sup> Recrystallizing solvent, A = dimethylformamide + acetonitrile, B = dimethylformamide, C = dimethylformamide + ether, D = acetonitrile. <sup>d</sup> Analyses by Weiler and Strauss, Oxford, England. <sup>e</sup> C<sub>6</sub>H<sub>5</sub>— is allyl. <sup>f</sup> R<sub>1</sub>R<sub>2</sub>N— is piperidino. <sup>g</sup> R<sub>1</sub> = CH<sub>2</sub>—, <sup>h</sup> Isolated as a hydrate. <sup>i</sup> Reported in Ref. 3. <sup>j</sup> R<sub>2</sub> = C<sub>2</sub>H<sub>5</sub>—.

## EXPERIMENTAL

**General Procedure for 2-Amino-4-substituted-amino-6-(ureidomethyl)-s-triazines.**—To 0.05 mole of the biguanide in 50 ml of methanol was added 0.05 mole of ethyl hydantoate and the mixture warmed to effect solution. After standing four days at 20° the reaction mixture was added to 50-100 ml of water. The precipitate of product (see Table I) was separated and recrystallized.

## REFERENCES

- (1) (a) Chiti, W., and Sella, R., *Farmaco Patia Ed sci*, 11, 607 (1956). (b) Beaver, D. J., Roman, D. P., and Stoeffel, P. J., *J. Am. Chem. Soc.*, 79, 1236 (1957). (c) Baltzly, R., Blackman, S. W., and Ide, W. S., *ibid.*, 76, 1165 (1954). (d) Cannon, J. G., and Webster, G. L., *This Journal*, 42, 740 (1953). (e) Spelman, M. A., Geisler, A. O., and Close, W. J., *J. Am. Chem. Soc.*, 70, 1189 (1948).
- (2) Goodman, L. S., and Gilman, A., "The Pharmacological Basis of Therapeutics," 2nd ed. The Macmillan Co., New York, N. Y., 1955, p. 197.
- (3) Shapiro, S. L., Parrino, V. A., and Freedman, L., *J. Am. Chem. Soc.*, 81, 3996 (1959).
- (4) Shapiro, S. L., Parrino, V. A., and Freedman, L., *ibid.*, 81, 3728 (1959).
- (5) Shapiro, S. L., Parrino, V. A., Rogow, E., and Freedman, L., *ibid.*, 81, 3725 (1959).
- (6) Smolin, E. M., and Rapoport, L., "s-Triazine and Derivatives," Interscience Publishers, New York, N. Y., 1959, pp. 56, 236.
- (7) Shapiro, S. L., Parrino, V. A., and Freedman, L., *J. Org. Chem.*, 25, 384 (1960).

\* Received May 17, 1960, from the Research Laboratories of the U. S. Vitamin & Pharmaceutical Corp., Yonkers 1, N. Y.

The authors are indebted to Dr. G. Ungar and his staff for the pharmacological screening of the compounds.



# Note on a Revised Sugar Coating Procedure for Tablets\*

By S. J. TUCKER and A. B. REDNICK

A method is presented which describes a revised sugar coating procedure. The procedure eliminates the rounding and smoothing operations usually employed in pan-coated tablets. The resulting coating is approximately one-half as thick as the standard method and coating time is greatly reduced.

TUCKER (1) described the use of insoluble colorants in the sugar coating of tablets. The method has two distinct advantages over the use of water-soluble dyes. The first is that the tablets cannot be overcolored, thus one does not have to be overly concerned about the number of color coats applied. The second is that the color does not have to be applied to perfectly smoothed tablets in order to achieve uniform distribution. These points led us to critically re-evaluate our present sugar coating methods.

In the standard sugar coating procedure a large part of the time is spent in sealing, rounding, and smoothing the tablet and in overcoming the white background during the coloring phase. Also, in the standard coating procedure, the operator must first go to the trouble of compressing a smooth tablet, must then rough it up in order to round the edges, and, finally, must smooth it out before color can be applied.

All these points were taken into consideration when revising the coating procedure employing pigments. The new procedure is divided into three basic phases: (a) The tablet is given two coats of acacia, using gelatin solution as the adhesive. This gives the tablet sufficient strength to withstand chipping and cracking in the coating pan and at the same time removes the sharp edges of the tablet. In addition, a colorant is added to the adhesive, thus, coloring begins immediately. (b) The tablet is sealed with one coat of a gum or resin cast from a suitable solvent. (c) The tablet is finished off using a pigment coating suspension.

## DEVELOPMENT AND PROCEDURE

A stock suspension (Table I) is prepared by combining the water, dioctyl sodium sulfosuccinate, and pigment in a Waring Blendor for fifteen minutes. This is then transferred to an Eppenbach Homomixer and, while mixing, the syrup and titanium

TABLE I.—TYPICAL COATING FORMULATION

Stock suspension, ingredients	
Purified water	250.00 ml.
Dioctyl sodium sulfosuccinate	0.01 Gm.
Insoluble colorant	1–15.00 Gm.
Titanium dioxide U. S. P.	0–100.00 Gm.
Syrup U. S. P.	500.00 ml.
Coating suspension	
Stock suspension	100.00 Gm.
Coating syrup <i>q. s.</i> ad.	500.00 ml.

dioxide are added. The solids usually disperse completely within fifteen minutes.

The stock suspension is then combined with gelatin adhesive solution and mixed with an Eppenbach Homomixer to form the undercoating adhesive suspension (Table II).

TABLE II.—UNDERCOATING FORMULATIONS

Gelatin adhesive solution	
Gelatin U. S. P.	800 Gm.
Purified water	6500 ml.
Acacia U. S. P.	600 Gm.
Sucrose U. S. P.	9500 Gm.
Undercoating adhesive suspension	
Stock suspension	10–20%
Gelatin adhesive solution	80–90%

The core tablets, to be coated, are placed in a conventional coating pan, the size depending upon the batch load. Any standard shaped tablet is suitable (except a flat face); however, a more spherical tablet and a thinner edged tablet facilitate the procedure. Undercoating adhesive suspension is then applied to the rolling tablets until they are evenly and completely wetted. When the mass becomes tacky, acacia powder is dusted on the tablets in the usual fashion. This procedure is repeated again. The tablets now have a color somewhat similar to the finished color, though mottled in appearance. It is interesting to note here that lakes give a definite color to the tablets at this stage, whereas a pigment will not. In order to overcome this, one may use, with the undercoating adhesive, a dye or a lake that gives a similar color (it is not necessary to duplicate the color) to the finished product.

The tablets are now sealed with a gum or resin cast from a suitable solvent. For example, a four-pound cut of shellac diluted with an equal quantity of isopropyl alcohol is satisfactory. One coat is usually sufficient.

Coating suspension (Table I) is now applied and, generally, 25 coats are sufficient. The tablets are finished off and polished in the usual fashion.

The finished product retains the same pharmaceutical elegance as when made by our present methods, yet has a coating of approximately one-half the thickness, and this procedure is accomplished in about one-half the time.

## REFERENCE

(1) Tucker, S., Nicholson, A. E., and Engelbert, H., *This Journal*, 47, 849 (1958).

\*Received August 16, 1960, from the Smith Kline and French Laboratories, Philadelphia 1, Pa.  
Accepted for publication September 23, 1960.

## Book Notices

*General Chemistry—Inorganic and Organic.* By GARTH L. LEE and HARRIS O. VAN ORDEN. W. B. Saunders Co., West Washington Square, Philadelphia 5, Pa., 1960. vi + 637 pp. 15.5 × 23.5 cm. Price \$7.25.

This book covers a wide field in simple, concise, positive statements. It could be useful as a textbook in the general science course. The author might have omitted the use of 2 stacked nickels to represent 1 cubic centimeter in the illustration of volume apparatus. It might be difficult for a chemistry minor to grasp the full text, but if he learned to use the book as a starting reference it could be stimulating. A good index is appended and the 1958 revision of atomic weights is included.

*Metal-Binding in Medicine* Edited by MARVIN J. SEVEN and L. AUDREY JOHNSON. J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa., 1960. xiii + 400 pp. 18 × 25.5 cm. Price \$13.75.

This book includes the papers and panel discussions from a symposium held May 6-8, 1959, in Philadelphia. The term "metal-binding" is used to indicate linkage between a binding agent and a metal, with "chelation" being reserved for the process of metal-binding in which the metal is incorporated into a ring structure. The material covers a wide field, starting with "The Relationship of Chemical Structure to Metal-Binding Action" and including pharmacological, physiological, clinical, and therapeutic considerations of natural and induced metal-binding forms in normal and pathological systems. An index is appended.

*Synthetic Methods of Organic Chemistry.* Vol. 14. By W. THEILHEIMER. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xvi + 549 pp. 15 × 23 cm. Price \$29.50.

Most of the references in Vol. 14 of this compilation of new methods for the synthesis of organic compounds and improvements of known methods cover papers published between 1957 and 1959. A comprehensive and concise reference series.

*An Introduction to the Chemistry of Heterocyclic Compounds.* By R. M. ACHESON. Interscience Publishers, Inc., 250 Fifth Ave., New York 1, N. Y., 1960. xiv + 342 pp. 15 × 23 cm. Price \$5.

This book attempts to present to the undergraduate student a concise account of the more important properties and chemical reactions of the basic heterocyclic systems. It is admittedly incomplete, omitting sugars and alkaloids, which the author assumes have been covered in other courses. Included are pertinent physical data, electronic and mechanistic concepts where possible, and brief discussions of chemical discoveries concerning metabolism and biosynthesis of some important compounds. Compound and subject indexes are appended.

*Einfache Versuche auf dem Gebiete der organischen Chemie.* 8th ed. By A. F. HOLLEMAN and LEONHARD SCHULER. Walter de Gruyter & Co., Genthner Strasse 13, Berlin W 35, Germany, 1960. xx + 172 pp. 14 × 22 cm. Paperbound.

This book is a teaching text (in German) of simple and basic chemical reactions of organic compounds and their functional groups.

*Analytische Trennung und Identifizierung organischer Substanzen.* By OTTO NEUNHOEFFER. Walter de Gruyter & Co., Genthner Strasse 13, Berlin W 35, Germany, 1960. xii + 116 pp. 17.5 × 23.5 cm. Price DM 18.

This book (in German) covers concisely the methods for analytical separation and identification of organic substances as applied in a teaching laboratory.

*Lehrbuch der organischen Chemie.* 35-36 ed. Edited by A. F. HOLLEMAN and FRIEDRICH RICHTER. Walter de Gruyter & Co., Genthner Strasse 13, Berlin W 35, Germany, 1960. xii + 646 pp. 17 × 24 cm. Price DM 28.

This classic textbook (in German) has been revised and edited again by Friedrich Richter who has performed this task for at least 30 years. Its reference value has been long established in the U. S. and elsewhere.

*Methods in Medical Research.* Vol. 8. Edited by H. D. BRUNER. The Year Book Publishers, Inc., 200 East Illinois St., Chicago, Ill., 1960. xiv + 368 pp. 13.5 × 21.5 cm. Price \$9.75.

The subjects considered in the eighth volume of this series are grouped under the main headings: Life history of the erythrocyte, Measurement of responses of involuntary muscle, and Peripheral blood flow measurement. Subject and name indexes are appended.

*Selective Toxicity.* By ADRIEN ALBERT. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. x + 233 pp. 14 × 21.5 cm.

Beginning with a discussion of the biochemical differences between species which introduce the possibility of selectivity into toxicity, four introductory paragraphs of this text are designed for undergraduate students in biochemistry, medicine, pharmacy, and agriculture. Nine other chapters take up advanced material related to biologically-active substances, including absorption and distribution, pharmacodynamics, covalency, surface chemistry, and steric factors. Appendix 1 is a tabulation showing the connection between ionization and antibacterial activity in the acridine series (101 compounds). Appendix 2 tabulates calculated percentage ionized, given pKa and pH (if anion or cation). Appendix 3 lists physical effects of organic substituents.

*Medicinal Chemistry* 2nd ed Edited by ALFRED BURGER Interscience Publishers, Inc, 250 Fifth Ave, New York 1, N Y, 1960 viii + 1243 pp 17.5 × 25 cm Price \$37.50

In the revision of his excellent treatise on medicinal chemistry, Professor Burger has enlisted the aid of 34 contributors. This was done in order to complete a comprehensive revision before portions of the material became out-dated. The expansion of the subject since the first edition of 1951 is indicated by an increase of 12 new chapters. The book is still designed for the more advanced reader and to point the direction of research needed in medicinal chemistry. The thoroughly splendid development and presentation of the text material follows the standard set in the two volumes of the first edition which were reviewed in *THIS JOURNAL*, 40, 167, 417(1951). A new two column format and slightly larger pages have enabled the increased material of the second edition to be published in one volume. The reference value of the book is enhanced by the adequate subject index.

*Pharmacology and Therapeutics* 4th ed By ARTHUR GROLLMAN Lea & Febiger, Washington Square, Philadelphia 6, Pa, 1960 1079 pp 15 × 23.5 cm Price \$12.50

Publication of the fourth edition of this book only two years after the third edition indicates the author's attempt to keep pace with the rapid changes, especially in therapeutics and drugs. Included are new sections on corticosteroid therapy, treatment of cancer, present day use and understanding of tranquilizers, the newer hormones, and psychic energizers. All the drugs introduced during the two years prior to the revision of the text are included. Designations of official preparations refer to U S P XI and N F X. It is a useful reference and textbook.

*Oxidation Reduction Potentials of Organic Systems* By W. MANSFIELD CLARK The Williams & Wilkins Co, 428 East Preston St, Baltimore 2, Md, 1960 xi + 584 pp 15 × 23 cm Price \$13.50

In referring to the usefulness of the material in this book, the author states "It should be clearly understood that when concepts of mechanisms and structures are used the purpose is to provide a neat organization of relations, confirmation of what is predicted by the final equations does not necessarily prove the postulates." More than 100 tables of data are included in this trustworthy text.

*Index Chemicus* Edited by EUGENE GARFIELD Institute for Scientific Information, 1122 Spring Garden St, Philadelphia 23, Pa, 1960 131 pp 21.5 × 28 cm Paperbound Issued monthly Price \$250 per year Educational, \$500 per year Industrial

A new (Vol 1, No 1) monthly index of new chemicals (3518 in this issue) includes listings of chemical names, structural diagrams, molecular formulas, and complete bibliographical references. Indexes will be cumulated quarterly and yearly.

*Recent Progress in Hormone Research* Vol 16 Edited by GREGORY PINCUS Academic Press Inc, 111 Fifth Ave, New York 3, N Y, 1960 viii + 608 pp 15 × 23 cm Price \$14

Reports on recent investigations presented at the 1959 Laurentian Hormone Conference are grouped under the main headings: Mechanisms of hormone action, Peptide hormones, Thyroid hormones, and Hormones and metabolism. Discussions of reports are included and author and subject indexes are appended.

*Précis de Chimie Biologique* Vol 2 By J. F. COURTOIS and R. PERLES Masson et Cie, 120, boulevard Saint Germain, Paris 6<sup>e</sup>, France, 1960 viii + 620 pp 16 × 21 cm Price 46 NF

Subjects covered in Vol 2 of this textbook are Lipides, Proteins, and Experimental functional exploration. A notice on Vol 1 appeared in *THIS JOURNAL*, 49, 182(1960).

*Galenisches Praktikum* By KURT MUNZEL, JAKOB BUCHI, and OTTO ERICH SCHULTZ Wissenschaftliche Verlagsgesellschaft m b H, Stuttgart, Germany, 1959 1114 pp 16 × 24 cm Price DM 142

The "Galenical Handbook" is a textbook (in German) on pharmacy. It is comprehensive in its coverage of procedures, with many examples of formulations included.

*The Chemistry of Heterocyclic Compounds* Vol 14 Pyridine and Its Derivatives Part 1 Edited by ERWIN KLINGSBERG Interscience Publishers, Inc, 250 Fifth Ave, New York 1, N Y, 1960 vi + 613 pp 15 × 23 cm Price \$49 Subscription \$42

Part one of a four-part monograph includes Properties and reactions of pyridine and its hydrogenated derivatives, by R. A. Barnes, and Synthetic and natural sources of the pyridine ring, by F. Brody and P. R. Ruby.

*Biochemistry of Plants and Animals* By M. FRANK MALLFETTE, PAUL M. ALTHOUSE, and CARL O. CLAGETT John Wiley & Sons, Inc, 440 Fourth Ave, New York 16, N Y, 1960 viii + 552 pp 15 × 23 cm Price \$8.50

A broad treatment of biochemistry is used to cover a wide variety of topics in the biological, physical, and agricultural sciences at the elementary level. This book replaces "Introduction to Agricultural Biochemistry" by Dutcher, Jensen, and Althouse (Wiley, 1951).

*Clinical Chemistry Principles and Procedures* 2nd ed By JOSEPH S. ANNINO Medical Book Department, Little, Brown & Co, 34 Beacon St, Boston 6, Mass, 1960 viii + 348 pp 15.5 × 23.5 cm Price \$8

A textbook for students of clinical chemistry on the basic technical level, the major part of the text (pp 75-339) is devoted to specific methods of clinical analysis.

# JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME 49

DECEMBER 1960

NUMBER 12

## New Approach to the Determination of Vitamin A in Pharmaceutical Products\*

By REAL TARDIF

A modified analytical procedure for the determination of vitamin A in pharmaceutical preparations and its method of calculation based on geometric and trigonometric relationships is presented. Ether and isopropyl alcohol solvents are replaced by hexane, a more selective solvent, for the extraction of the unsaponifiable fraction. Absorbance readings of this latter are measured at one wavelength ( $325\text{ m}\mu$ ) before and after the destruction of vitamin A. A comparison study of the accuracy and precision of the U. S. P. XV method and of this proposed method was made on five characteristic polyvitamin preparations and on a cod liver oil sample. Statistical analysis of the results in per cent recovery of added vitamin A has shown a 100 per cent yield by the "hexane-destruction" method, and 82 to 96 per cent yield by the U. S. P. XV method. With this proposed method, the standard deviations were less than one-third as great.

**D**URING the last decade a great variety of vitamin A combinations has appeared on the market. To estimate the vitamin A potency of these complex mixtures, the analyst has the choice of biological (1), chemical (2), and physico-chemical methods (3-7). The biological assay is unfortunately impractical for routine control of vitamin A because of the cost, the time required, and the wide variation in results.

Chemical and physico-chemical methods, which are most practical, are of value as long as they assess the true physiological activity of products under investigation. In our experience it is very seldom that the official physico-chemical method for vitamin A with the Morton-Stubbs correction (3, 4) can be applied without over-correcting, even if a part of the interfering material has been removed by saponification. As the essence of this correction is the linearity

of the irrelevant absorbance at the chosen wavelengths, limitation in its application can be foreseen (3, 8-12).

Our analytical procedure employs a more selective solvent, hexane, and also a mathematical correction, the essence of which is a comparison of the absorbance readings at one wavelength before and after the vitamin A in the unsaponifiable fraction has been destroyed.

The reliability of results obtained by our method is dependent on this condition: when a Canadian Reference Standard capsule of vitamin A is treated as an unknown and the conversion factor of 1,870, determined in our laboratory, is applied, the stated potency of 25,000 I. U./capsule must be found when the Morton-Stubbs correction (for hexane) is applied (Eq. 4).

### ANALYTICAL PROCEDURE

**Reagents.**—Hexane 72-1,005 from Shell Oil Co. purified as follows: 1,500 ml. is shaken twice for five-minute periods with 120-ml. portions of concentrated sulfuric acid; wash with 200 ml. of water,

\* Received March 9, 1960, from the Research Laboratories of Frank W. Horner Ltd., P. O. Box 959, Montreal, Canada. Accepted for publication June 29, 1960.  
Thanks are due to Dr. R. W. Lehman, Vitamin and Development Laboratory, Distillation Products Industries, for useful suggestions, and also to Antonio LaTorre for help in experimental work.

then with 200 ml of 1% sodium hydroxide, and finally with 200 ml of water, shake two minutes each time, distill hexane slowly and collect portions between 67 and 70°, dry over anhydrous sodium sulfate. Sodium sulfate reagent grade. Potassium hydroxide 50% w/v aqueous solution, reagent grade.

**Saponification.**—Transfer to a saponification flask a suitable quantity of sample (size depends upon potency, 10 I U/ml wanted in final dilution). Boil under reflux, in an all-glass apparatus, with 5 ml of potassium hydroxide 50% w/v, (3 to 7 ml, or more, depending on sample) and 30 to 40 ml of ethyl alcohol for thirty minutes. Use electric hot plate and adjust heat to minimum required to have a good boiling. Cool the flask under tap water and transfer solution to a 250 ml Squibb type separatory funnel. Rinse saponification flask completely with 3 × 10 ml portions of water. The aqueous solution is extracted with 1 × 40 ml and 3 × 30 ml (or more) portions of purified hexane. The hexane portions are collected in one separatory funnel. Wash hexane extracts with 50-ml portions of water. Repeat until the wash water gives no color with phenolphthalein (4 to 5 washings). Using a small funnel and a cotton plug, transfer the hexane extraction into a volumetric flask of not less than 200 ml. Rinse with purified hexane and make up to volume.

**Destruction Step.**—Transfer 50 ml of hexane extract to a 150 ml separatory funnel. Transfer also 50 ml of purified hexane to another 150 ml separatory funnel to serve as a reagent blank for the destruction of vitamin A. Add to each separatory funnel 10 ml of 60% sulfuric acid and shake 150 times. Discard sulfuric acid and repeat with another 10 ml of fresh sulfuric acid, or until sulfuric acid is colorless. Wash three times with 15 ml of distilled water (shake about 25 times). Filter through a cotton plug into an Erlenmeyer flask.

**Spectrophotometric Readings.**—Readings are made on a Beckman DU spectrophotometer. The absorbance of the Canadian Reference Standard is read against purified hexane at 310, 325, and 334 mμ. For the samples (undestroyed hexane extract), the readings are made at 325 mμ only, however, if read at 310 and 334 mμ, the analyst can also apply the Morton-Stubbs correction (Eq 4). The absorbance of the "destroyed hexane extract" is read against the purified hexane submitted to the destruction procedure (reagent blank). All readings are made at 325 mμ only.

**Comparison of Analytical Procedures.**—A comparison of the analytical operations in both methods is made in Table I.

Hexane replaces the ether and isopropyl alcohol and also eliminates the evaporation of an ether aliquot in an inert atmosphere. Absorbance readings are measured at one wavelength instead of at three. Destruction of vitamin A is an extra operation.

## THEORETICAL

**Pure Vitamin A Standard.**—If one can imagine a right angle triangle C'D'B' (Fig 1) in which the length of the base B'D' corresponds to the absorbance reading of the vitamin A standard (A std) at 325 mμ and the length of the height C'D' corresponds to

TABLE I—COMPARISON OF ANALYTICAL PROCEDURES

Analytical Operations	Methods	
	U S P	Hexane Destruction
Saponification	Yes	Yes
Extraction solvent	Ether	Hexane
Evaporation of solvent in nitrogen	Yes	No
Dissolution of residue in isopropyl alcohol	Yes	No
Make to volume with solvent	Isopropyl alcohol	Hexane
Destruction of vitamin A	No	Yes
Absorbance readings	310, 325, 334 mμ	325 mμ
Calculation	Equation 5	Equation (std) Equation (sample)

the absorbance reading of the same vitamin A standard at the same wavelength after it has been subjected to the vitamin A destruction procedure (dA std), the ratio of these two experimental readings or these two sides will be equal to the value of the tangent of the angle α

$$\text{tangent } \alpha = \frac{C'D'}{B'D'} = \frac{\text{dA std}}{\text{A std}} \quad (\text{Eq 1})$$

**Sample.**—As in the case of pure vitamin A standard one can also visualize another right angle triangle GDE (Fig 2) in which the right angle triangle CDB (Fig 1) can also be included. Similarly, the length of the base ED can correspond to the total absorbance at 325 mμ of the sample in this instance being composed of BD or A and EE

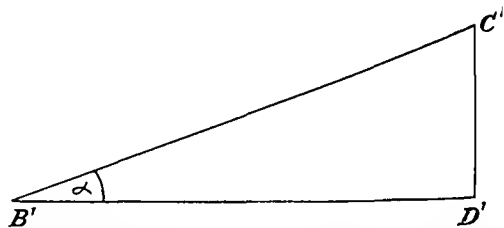


Fig 1—Pure vitamin A. B'D', absorbance of undestroyed standard (A std), C'D', absorbance of destroyed standard (dA std)

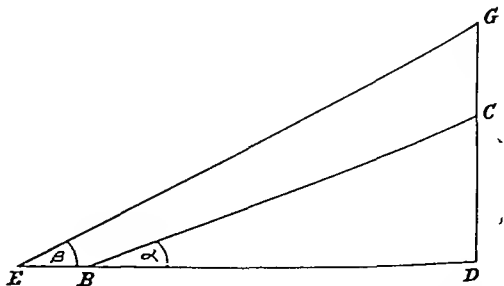


Fig 2—Sample ED, absorbance of undestroyed sample (BD or A) + (EB or F) GD, absorbance of destroyed sample (CD or DA) + (GC or F)

or F, respectively, the absorbance of pure vitamin A in the sample and the absorbance of all the foreign materials in the same sample. Also similarly, the length of the height GD can correspond to the total absorbance at 325  $m\mu$  of the sample once it has been subjected through the vitamin A destruction procedure; CD or dA and GC or dF being, respectively, the absorbance at 325  $m\mu$  of the destroyed pure vitamin A and the destroyed foreign materials originally present in the sample. The ratio of these two experimental values, or two sides, will be equal to the value of the tangent of the new angle  $\beta$ .

$$\text{tangent } \beta = \frac{GD}{ED} = \frac{CD + GC}{BD + EB} = \frac{dA + dF}{A + F} = \frac{\text{absorbance destroyed sample}}{\text{absorbance sample}} \quad (\text{Eq. 2})$$

The object is now to calculate the value of BD (Fig. 2), the corrected absorbance for pure vitamin A in the sample under investigation. Use is made of four values either obtained or calculated from experimental readings.

base ED = absorbance of sample at 325  $m\mu$   
height GD = absorbance of sample at 325  $m\mu$   
after destruction

$$\text{tangent } \alpha = \frac{CD}{DB} = \frac{\text{absorbance of standard at 325 } m\mu}{\text{absorbance of standard at 325 } m\mu \text{ after destruction}}$$

$$\text{tangent } \beta = \frac{GD}{ED} = \frac{\text{absorbance of sample at 325 } m\mu}{\text{absorbance of sample at 325 } m\mu \text{ after destruction}}$$

Derivation of the following simplified expression to calculate the value of BD was made with the help of geometric and trigonometric relationships

$$A_{325} \text{ corr.} = BD = \frac{GD (1 + \tan^2 \alpha)}{\tan \beta (1 + \tan^2 \beta)} \quad (\text{Eq. 3})$$

## EXPERIMENTAL

Reports in the literature (13-17) indicate that solvents, ethyl ether and petroleum ether, were successfully employed for a complete extraction of the unsaponifiable fraction. The difference between the purified hexane employed in our work and petroleum ether used by others is not very great; this latter is a mixture of pentane and hexane hydrocarbons while the former is pure normal hexane with traces only of methyl pentane, both saturated hydrocarbons (18). These authors pointed out certain disadvantages in using these solvents such as formation of peroxides in the ethyl ether, appearance of persistent emulsions, and partial extraction of some of the soaps. With pure hexane these are eliminated.

**Morton-Stubbs Correction Applied to Hexane Solvent.**—On different days five Canadian reference standard capsules (25,000 I. U./capsule) were saponified and the unsaponifiable fraction extracted as outlined in the hexane destruction analytical procedure. The ratios ( $1/K'$  and  $1/K^2$ ) of the absorbance readings recommended for the Morton-

TABLE II.—VALUE OF  $1/K'$  AND  $1/K^2$  FOR PURE VITAMIN A IN HEXANE

Ratios	Mean Value of Ratio	Standard Deviation
$1/K'$ or $A_{310}/A_{325}$	0.8455	$\pm 0.0050$ or $\pm 0.6\%$
$1/K^2$ or $A_{334}/A_{325}$	0.8745	$\pm 0.0026$ or $\pm 0.3\%$

Stubbs correction (3) were calculated and analyzed statistically (Table II).

Substituting the above experimental values for  $1/K'$  and  $1/K^2$  into the Morton-Stubbs general formula, the following equation is obtained

$$A_{325} (\text{corr.}) = \frac{3(A_1 - A_2) + 5(A_1 - A_3)}{1.091 \pm 0.028} \quad (\text{Eq. 4})$$

For ether and isopropyl alcohol, the U. S. P. formula is very close

$$A_{325} (\text{corr.}) = \frac{3(A_1 - A_2) + 5(A_1 - A_3)}{1.172} \quad (\text{Eq. 5})$$

**Selectivity of Hexane Solvent for the Extraction of the Unsaponifiable Fraction.**—Two samples of a blank (no vitamin A) polyvitamin preparation were saponified and the unsaponifiable fraction of one sample was extracted with ether while the other was extracted with hexane. Results of the absorbance readings at 310, 325, and 334  $m\mu$  are given in Table III.

The absorbance of the extraneous material extracted from the blank polyvitamin preparation is much greater when extracted with ether than with hexane, especially at 310  $m\mu$ . With hexane, the absorbance readings are much closer to linearity than when extracted with ether. In terms of over-correction at 325  $m\mu$ , the ether represents a drop of 15%, or three times greater than with hexane.

Two samples of polyvitamin products were also saponified and extracted as above with two solvents, ether and hexane. Results of the absorbance readings are given in Table IV.

With hexane, the absorbance readings of the polyvitamin preparations are also smaller than when extracted with ether. In terms of yields, the hexane-destruction method shows a recovery of 100% while the Morton-Stubbs correction gives only 85%.

**Comparison between the Morton-Stubbs and Hexane-Destruction Methods.**—Five characteristic polyvitamin formulations (elixirs, emulsions, drops, capsules with, and without minerals) were prepared similar to those on the market with the exception of vitamin A. A two-year old sample of cod liver oil was also included in this study.

To a measured quantity of each of the above preparations, a capsule of Canadian Reference Standard vitamin A (25,000 I. U./capsule) was added to the saponification flask just prior to saponification. The cod liver oil sample was analyzed before and after the addition of a known amount of the same Canadian Reference Standard vitamin A to the saponification flask. Each analysis was repeated six times for the polyvitamin preparations and four times for the cod liver oil sample. The results, which have been expressed in % recovery for the polyvitamin preparations, and in I. U./Gm for the cod liver oil sample, were calculated by both the Morton-Stubbs (Eq. 4) and

TABLE III —SELECTIVITY OF HEXANE SOLVENT ON BLANK POLYVITAMIN PREPARATION

Solvents	Absorbance $\mu\mu$			Absorbance Expected for Linearity at 310 $\mu\mu$	Overcorrection—	
	310	325	334		Absorbance	% at 325 $\mu\mu$
Ether-isopropanol	0 045	0 021	0 018	0 0255	0 0831	14 5
Hexane	0 028	0 014	0 009	0 022	0 0256	4.65

TABLE IV.—SELECTIVITY OF HEXANE SOLVENT ON A POLYVITAMIN PREPARATION

Solvents	Absorbance, $\mu\mu$			Method	Absorbance at 325 $\mu\mu$ Corr	% Yield on Theoretical Claim
	310	325	324			
Ether-isopropanol	0 516	0 575	0 500	M. S <sup>a</sup>	0.471	83.7
Hexane	0 481	0 552	0 492	M S <sup>b</sup>	0.469	85 2
				H. D <sup>c</sup>	0.552	100.0

<sup>a</sup> Morton Stubbs correction for isopropyl alcohol (Eq 5)    <sup>b</sup> Morton Stubbs correction for hexane (Eq. 4).    <sup>c</sup> Hexane destruction correction (Eq 3)

TABLE V —STATISTICAL COMPARISON OF PER CENT VITAMIN A RECOVERY CALCULATED BY THE MORTON STUBBS AND HEXANE-DESTRUCTION METHODS

Products	I U Vitamin A Added	No of Assays	% Recovery—		Experimental Morton- Stubbs	Value Hexane Destruction
			Morton Stubbs Mean % ± S D	Hexane- Destruction Mean % ± S D		
Canadian Ref Std., 25,000 I. U./capsule		5	99 9 ± 0 3		0 6 <sup>b</sup>	
Elixir A	179/ml	6	82 2 ± 1 7	99 9 ± 0 4	22 4 <sup>c</sup>	0 2 <sup>b</sup>
Elixir B	200/ml	6	86 1 ± 3 9	99 7 ± 0 6	7 9 <sup>c</sup>	0 7 <sup>b</sup>
Emulsion C	1,087/ml	6	95 5 ± 3 0	100 1 ± 0 2	3 2 <sup>c</sup>	1 2 <sup>b</sup>
Drops D	12,500/ml	6	93 5 ± 2 2	100 2 ± 0 1	6 5 <sup>c</sup>	2 2 <sup>b</sup>
Capsules E	11,364/capsule	6	96 5 ± 3 6	100 1 ± 0 3	2 1 <sup>b</sup>	1 2 <sup>b</sup>

<sup>a</sup> For a probability  $P > 0.05$ , the  $t$  value  $> 2.26$   
<sup>b</sup> Experimental  $t > 2.26$  = no significant difference between % recovery and the vitamin A in sample.  
<sup>c</sup> Experimental  $t < 2.26$  = significant difference between % recovery and the vitamin A in sample

hexane-destruction (Eq 3) methods of correction before being statistically analyzed (Tables V, VI, VII)

The hexane-destruction method of calculation showed no significant difference between the per cent recovery and the amount of vitamin A added to these samples. In four samples the Morton-Stubbs method of calculation showed a significant difference between the per cent recovery and the amount of vitamin A added.

TABLE VI.—POTENCY OF COD LIVER OIL SAMPLE: STATISTICAL ANALYSIS OF RESULTS CALCULATED BY THE MORTON-STUBBS AND HEXANE-DESTRUCTION METHODS

Methods	Morton-Stubbs	Hexane Destruction
1st Assay	2,076 I. U./Gm.	2,406 I. U./Gm.
2nd Assay	2,107 I. U./Gm.	2,399 I. U./Gm.
3rd Assay	1,902 I. U./Gm.	2,369 I. U./Gm.
4th Assay	2,172 I. U./Gm.	2,329 I. U./Gm.
Average	2,064 I. U./Gm.	2,376 I. U./Gm.
Standard deviation	±105	±36
Confidence limits	±193	±66
$t$ Value ( $P = 0.05, n = 4$ )		3.18
$t$ Value (experimental)		7.15

An experimental  $t$  value of 7.15 at this probability level ( $P = 0.05$  and  $n = 4$ ) indicates that the result obtained by both methods of calculation are significantly different. The standard deviation and the confidence limits are only one-third as great when calculated from the hexane-destruction results.

TABLE VII.—STATISTICAL COMPARISON OF PER CENT RECOVERY OF ADDED VITAMIN A TO COD LIVER OIL: MORTON-STUBBS vs. HEXANE-DESTRUCTION METHODS

I U/Gm Added Vitamin A	—Morton Stubbs—		Hexane Destruction	
	I U/Gm	Recovery, %	I U/Gm	Recovery, %
1,459	1,264	86 6	1,361	93.3
1,283	1,005	78 3	1,257	98 0
1,326	1,534	115 7	1,415	106 7
1,148	1,204	104 9	1,177	102 5
Average	1,304	96 6	1,303	100.1
Standard deviation	±111			
Confidence Limits	±189	±14.7	±92	±5.0
( $P = 0.05$ and $n = 4$ )	±204	±27 0	±169	±9 2

The average per cent recovery of added vitamin A is very good: 100.1% by the hexane-destruction method and 96.6% by the Morton-Stubbs. At this probability level, the standard deviation and the confidence limits are only one-third as great when calculated from the hexane-destruction results.

With the same experimental results, the per cent recovery of added vitamin A into the same blank polyvitamin preparations was also calculated by a blank correction method: absorbance of sample at 325 m $\mu$  less absorbance of destroyed sample. Comparison of these results with those obtained by the hexane-destruction method of calculation is made in Table VIII.

TABLE VIII.—COMPARISON OF THE PER CENT VITAMIN A RECOVERY: BLANK CORRECTION METHOD vs. HEXANE-DESTRUCTION METHOD

Products	I. U Vitamin A Added	% Recovery		Difference in % Recovery
		Hexane Destruction	Blank Correction	
Elixir A	179	99.9	94.1	5.8
Elixir B	200	99.7	93.9	5.8
Emulsion C	1,087	100.1	96.4	3.7
Drops D	12,500	100.2	97.9	2.3
Capsule E	11,364	100.1	98.4	1.7

TABLE IX.—COMPARISON OF A SINGLE HEXANE EXTRACTION vs. THE HEXANE-DESTRUCTION METHOD

Products	Curr Absorbance at 325 m $\mu$		Difference, %
	Hexane- Destruction Method	Single Hexane Extn	
Low vitamin A elixir, 200 I. U./ ml.)	0.636	0.628	-1.3
High vitamin A drops, 12,500 I. U./ml.	0.619	0.604	-2.4

With high vitamin A potency preparations (drops D and capsule E) both methods of calculation are acceptable. There is less than 2% difference in per cent recovery. With the low vitamin A potency preparations (elixir A and B, emulsion C) the difference between the per cent recovery of both methods is two to three times greater.

The suggestion of Napoli, *et al* (13), of replacing

all extractions by a single extraction with the same total volume of solvent was investigated with the hexane solvent on a low and a high vitamin A potency polyvitamin preparation (Table IX).

This extraction modification is certainly acceptable for routine control determinations, especially if the analyst considers the small per cent difference in the vitamin A extraction between the two methods and the time required for the analysis, which is appreciably reduced.

## SUMMARY

A modified analytical procedure for determining vitamin A in pharmaceutical preparations is described. A new method of calculation based on geometric and trigonometric relationships is proposed.

The hexane-destruction method of calculation was applied to five polyvitamin products and a cod liver oil sample to which known amounts of vitamin A standard were added. Statistical analysis of the results showed greater accuracy than obtained with the Morton-Stubbs method of calculation. Napoli's extraction modification is also applicable.

## REFERENCES

- (1) Fugsley, L. I., Wills, G., and Crandall, W. A., *J. Nutrition*, **28**, 365(1944)
- (2) Carr, F. H., and Price, E. A., *Biochem. J.*, **20**, 498 (1926)
- (3) Morton, R. A., and Stubbs, A., *Analyst*, **71**, 348(1946)
- (4) "United States Pharmacopeia," 15th Rev., Mack Publishing Co., Easton, Pa., 1955, p. 942, *ibid*, 2nd Suppl., p. 22
- (5) McGillivray, W. A., *Ind Eng Chem. Anal. Ed.*, **22**, 494(1950)
- (6) Oser, B. L., *ibid*, **21**, 529(1949)
- (7) Prnkhoznik, S. J., *Analyst*, **77**, 185(1952)
- (8) Griggeman, N. T., *ibid*, **76**, 449(1951)
- (9) Bagnall, H. H., and Stuck, F. G., *ibid*, **77**, 356(1952)
- (10) Marjaoui, A., and Gaudiauo, A., *Food Technol.*, **5**, 29(1951)
- (11) Canna, H. R., Collins, F. D., and Morton, R. A., *Biochem. J.*, **50**, 48(1951)
- (12) Matchett, J. R., and Von Loesekie, H. W., *Ind Eng Chem. Anal. Ed.*, **25**, 26(1953)
- (13) Napoli, J. A., Seoknyski, B. Z., and Motchane, A. E., *THIS JOURNAL*, **48**, 611(1959)
- (14) Mahann, J. H., and Chapman, R. A., *Anal. Chem.*, **26**, 1195(1954)
- (15) Schmall, M., Senkowski, B., Colarusso, R., Wollish, E. G., and Shafer, E. G. E., *ibid*, **47**, 839(1958)
- (16) Wilkie, J. B., Jones, S. W., and Kline, O. L., *ibid*, **47**, 385(1958)
- (17) Windham, E. S., *J. Assoc. Offic. Agr. Chemists*, **40**, 522(1957)
- (18) Private communication, Shell Oil Co. of Canada



# Synthesis of Some Symmetrical Aldehyde Glycol Monoether Acetals\*

By ARTHUR J. GETZKIN† and WERNER M. LAUTER

Forty-two acetals of seven aldehydes and the mono-methyl, ethyl and *n*-butyl ethers of ethylene glycol and diethylene glycol were prepared, and their physical constants determined. An *in vitro* test was used to determine their hydrolysis in a slightly acidic medium, considered to be similar to the natural acidity of the human skin. It was shown that regeneration of the parent aldehydes proceeded with sufficient rapidity to warrant *in vivo* tests of these acetals in cosmetic formulas.

ALDEHYDES REPRESENT some of the most valuable perfuming and flavoring agents. A major disadvantage of the class is their relative instability; their tendency toward various condensation and polymerization reactions which are frequently potentiated by light, heat, air, alkali, and amines. This usually results in loss of odor, change in fragrance, discoloration, or precipitation.

Acetals are frequently utilized because they retain the aromatic properties of the parent aldehydes and possess a good degree of stability. However, acetals are easily hydrolyzed in slightly acidic media, reverting to the respective aldehyde and alcohol components. The use of higher molecular weight alcohols in acetal synthesis has not received much attention because of the low volatility and consequent loss of aroma such acetals would possess. High molecular weight acetals have been noted as potential aromatic stabilizers or fixatives (1, 2).

Synthesis of the acetal of diethylene glycol ethyl ether was first discussed by Nieuwland (3). Glyoxal tetra acetals of the glycol monoethers have been prepared and their applications in cosmetics mentioned by MacDowell and McNamee (4).

The objective of this investigation was the preparation of symmetrical acetals of the monoethers of ethylene glycol and diethylene glycol which, while themselves of little olfactory value, would be easily hydrolyzed in slightly acidic media to the corresponding aldehydes. The original aldehyde would thus be restored with the glycol ether free to act as a fixative. The glycol ethers appear to fulfill the requirements for a cosmetic fixative, as expressed by Neurath (5).

Kulka (6) has stated that the reactivity and conversion rates of various aldehydes and al-

cohols depend upon their molecular weights and configurations. The following rules are generally accepted: (a) The conversion rate for primary alcohols is slow, but yields are high. (b) Secondary and tertiary alcohols react more rapidly, but yields are lower. (c) Polyhydric alcohols react satisfactorily and almost completely, especially with aromatic aldehydes. (d) Aliphatic and arylalkyl aldehydes react more slowly but more completely than aromatic aldehydes.

Aldehydes possessing a hydroxyl group do not lend themselves to direct acetylation due to the nature of the condensation which must occur. Synthesis of the acetals of such important oxyaldehydes as vanillin, *p*-hydroxybenzaldehyde, and salicylaldehyde is more complex and will be the subject of a future report.

## EXPERIMENTAL

The mono-methyl, ethyl, and *n*-butyl ethers of ethylene glycol and diethylene glycol were employed as alcohols; *n*-butyraldehyde, isodecaldehyde (mixed isomers), benzaldehyde, cinnamaldehyde, anisaldehyde, cuminal, and piperonal as aldehyde components for the acetals.

Concentrated hydrochloric acid, gaseous hydrogen chloride, concentrated sulfuric acid, concentrated phosphoric acid, anhydrous calcium chloride, and a cationic exchange resin<sup>1</sup> in catalytic quantities, were tried as condensing agents. Considerable resin formation and low acetal yields resulted. Experiments indicated that *p*-toluenesulfonylchloride would be an effective condensation agent and the use of this material did furnish satisfactory yields.

Between twenty-four and thirty hours of refluxing was necessary to adequately remove all water of reaction. The reaction mixture was quickly neutralized with sodium methoxide, which appeared more complete than the classical treatment with sodium carbonate.

The general preparative procedure for the compounds reported is as follows: The aldehyde, glycol ether, and *p*-toluenesulfonylchloride, in quantities representing a molar ratio of 3:5:0.001, respectively, were thoroughly mixed until the chloride was entirely dissolved. The mixture was allowed to stand for fifteen minutes and 40 ml. of water-saturated

\* Received February 19, 1960, from the College of Pharmacy, University of Florida, Gainesville.

This paper is based in part upon a dissertation presented to the Graduate Council of the University of Florida by Arthur J. Getzkin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

† Present address: Naval Medical Research Center, Bethesda, Md.

<sup>1</sup> Amberlite IR-120, Rohm and Haas Co.

TABLE I—ACETALS OF ETHYLENE GLYCOL-MONOETHERS WITH ALDEHYDES

Aldehyde	R <sub>1</sub>	Boiling Range, °C/mm	Refr Index 25°	Sp Gr 25°	Mol Wt	Mol Formula	C Calcd %	C <sup>a</sup> Found %	H Calcd, %	H Found %	Color	Yield %
<i>n</i> -Butyraldehyde	CH <sub>3</sub>	66-68/0 3	1 4205	0 9706	206 28	C <sub>10</sub> H <sub>22</sub> O <sub>4</sub>	58 22	58 43	10 75	10 48	Light yellow	53 0
	C <sub>2</sub> H <sub>5</sub>	80-86/0 25	1 4185	0 9226	234 33	C <sub>12</sub> H <sub>26</sub> O <sub>4</sub>	61 5	61 45	11 18	10 94	Colorless	57 4
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	110-116/0 25	1 4268	0 9025	290 43	C <sub>16</sub> H <sub>34</sub> O <sub>4</sub>	66 16	66 42	11 80	11 69	Light yellow	55 8
Isodecylaldehyde <sup>b</sup>	CH <sub>3</sub>	106-110/0 2	1 4340	0 9425	290 43	C <sub>16</sub> H <sub>34</sub> O <sub>4</sub>	66 16	66 69	11 80	11 99	Colorless	66 8
	C <sub>2</sub> H <sub>5</sub>	118-121/0 2	1 4330	0 9016	318 48	C <sub>18</sub> H <sub>38</sub> O <sub>4</sub>	67 88	68 16	12 03	12 39	Colorless	70 3
	<i>n</i> -C <sub>10</sub> H <sub>21</sub>	148-151/0 25	1 4355	0 8918	374 59	C <sub>22</sub> H <sub>46</sub> O <sub>4</sub>	70 54	70 59	12 38	12 49	Colorless	62 5
Benzaldehyde	CH <sub>3</sub>	102-106/0 2	1 4815	1 0812	240 29	C <sub>12</sub> H <sub>20</sub> O <sub>4</sub>	64 98	65 13	8 39	8 31	Colorless	53 6
	C <sub>2</sub> H <sub>5</sub>	132-135/0 75	1 4735	1 0084	268 34	C <sub>14</sub> H <sub>24</sub> O <sub>4</sub>	67 13	67 25	9 02	9 05	Colorless	58 4
	<i>n</i> -C <sub>10</sub> H <sub>21</sub>	164-170/1	1 4685	0 9719	324 45	C <sub>18</sub> H <sub>32</sub> O <sub>4</sub>	70 33	69 65	9 94	9 80	Colorless	57 9
Cinnamaldehyde	CH <sub>3</sub>	130-136/0 25	1 5160	1 0785	266 33	C <sub>14</sub> H <sub>22</sub> O <sub>4</sub>	67 64	67 78	8 33	8 02	Yellow	53 7
	C <sub>2</sub> H <sub>5</sub>	144-148/0 25	1 4988	1 0149	294 38	C <sub>16</sub> H <sub>26</sub> O <sub>4</sub>	69 36	69 13	8 90	8 92	Yellow	66 3
	<i>n</i> -C <sub>10</sub> H <sub>21</sub>	168-171/0 25	1 4917	0 9788	350 48	C <sub>20</sub> H <sub>38</sub> O <sub>4</sub>	71 96	71 91	9 78	9 70	Yellow	37 8
Anisaldehyde	CH <sub>3</sub>	132-137/0 3	1 4910	1 1117	270 32	C <sub>14</sub> H <sub>22</sub> O <sub>4</sub>	62 20	62 73	8 20	8 10	Colorless	61 4
	C <sub>2</sub> H <sub>5</sub>	140-144/0 3	1 4830	1 0477	298 37	C <sub>16</sub> H <sub>26</sub> O <sub>6</sub>	64 40	64 09	8 78	8 42	Colorless	65.6
	<i>n</i> -C <sub>10</sub> H <sub>21</sub>	166-170/0 3	1 4775	1 0039	354 47	C <sub>20</sub> H <sub>38</sub> O <sub>6</sub>	67 76	67 19	9 67	9 66	Colorless	57.5
Cuminal	CH <sub>3</sub>	124-127/0 25	1 4810	1 0388	282 37	C <sub>14</sub> H <sub>20</sub> O <sub>4</sub>	68 05	67 47	9 28	9 14	Colorless	51 1
	C <sub>2</sub> H <sub>5</sub>	134-138/0 25	1 4755	0 9853	310 42	C <sub>16</sub> H <sub>26</sub> O <sub>4</sub>	69 04	70 55	9 74	9 52	Colorless	58 1
	<i>n</i> -C <sub>10</sub> H <sub>21</sub>	162-166/0 25	1 4720	0 9548	366 52	C <sub>22</sub> H <sub>38</sub> O <sub>4</sub>	72 09	72 11	10 45	10 29	Colorless	63 2
Piperonal	CH <sub>3</sub>	142-146/0 25	1 5038	1 1855	289 3	C <sub>14</sub> H <sub>20</sub> O <sub>6</sub>	59 14	59 17	7 09	7 30	Light yellow	49 0
	C <sub>2</sub> H <sub>5</sub>	150-154/0 25	1 4925	1 1247	312 35	C <sub>16</sub> H <sub>22</sub> O <sub>6</sub>	61 52	61 90	7 75	7 91	Light yellow	49 0
	<i>n</i> -C <sub>10</sub> H <sub>21</sub>	172-177/0 25	1 4850	1 0686	368 46	C <sub>20</sub> H <sub>32</sub> O <sub>6</sub>	65 19	65 77	8 75	8 89	Light yellow	50 1

<sup>a</sup> All analyses were performed by Weiler and Strauss Microanalytical Laboratory Oxford England<sup>b</sup> Mixed isomers

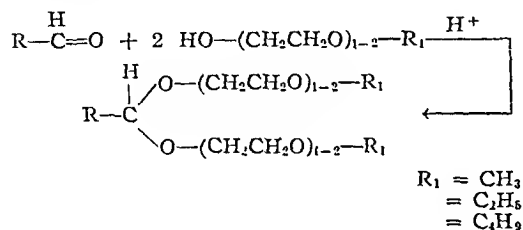
TABLE II —ACETALS OF DIETHYLENEGLYCOL-MONOETHERS WITH ALDEHYDES

Aldehyde	R <sub>1</sub>	Boiling Range, °C/mm	Refr Index, 25°	Sp Gr, 25°	Mol Wt	Mol Formula	C Calcd, %	C Found %	H Calcd %	H Found %	Color	Yield, %
<i>n</i> -Butylaldehyde	CH <sub>3</sub>	120-123/0 25	1 4345	1 0205	294.38	C <sub>14</sub> H <sub>30</sub> O <sub>6</sub>	57.12	57.13	10.27	10.24	Yellow	57.1
	C <sub>2</sub> H <sub>5</sub>	132-136/0 25	1 4350	0 9658	322.43	C <sub>16</sub> H <sub>34</sub> O <sub>6</sub>	59.60	59.68	10.63	10.40	Yellow	56.7
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	156-162/0 25	1 4382	0 9472	378.54	C <sub>20</sub> H <sub>42</sub> O <sub>6</sub>	63.45	63.82	11.18	10.95	Yellow	40.6
Isodecaldehyde	CH <sub>3</sub>	153-162/0 3	1 4390	0 9857	378.54	C <sub>20</sub> H <sub>42</sub> O <sub>6</sub>	63.45	63.43	11.18	11.21	Colorless	59.5
	C <sub>2</sub> H <sub>5</sub>	165-168/0 3	1 4375	0 9393	406.59	C <sub>22</sub> H <sub>46</sub> O <sub>6</sub>	64.98	65.37	11.40	11.47	Colorless	62.7
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	184-190/0 25	1 4432	0 9237	462.69	C <sub>26</sub> H <sub>54</sub> O <sub>6</sub>	67.49	67.41	11.76	12.48	Colorless	63.1
Benzaldehyde	CH <sub>3</sub>	156-160/0 3	1 4780	1.1000	328.39	C <sub>17</sub> H <sub>34</sub> O <sub>6</sub>	62.17	61.95	8.59	8.34	Colorless	56.3
	C <sub>2</sub> H <sub>5</sub>	168-172/0 35	1 4766	1.0376	356.45	C <sub>19</sub> H <sub>42</sub> O <sub>6</sub>	64.02	64.96	9.05	9.08	Colorless	41.2
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	194-198/0 3	1 4688	0 9982	412.55	C <sub>23</sub> H <sub>50</sub> O <sub>6</sub>	66.95	66.81	9.77	9.83	Colorless	52.7
Cinnamaldehyde	CH <sub>3</sub>	178-181/0 3	1 4980	1 0883	354.43	C <sub>19</sub> H <sub>30</sub> O <sub>6</sub>	64.38	64.85	8.53	8.65	Yellow	44.3
	C <sub>2</sub> H <sub>5</sub>	184-190/0 3	1 4950	1 0341	382.48	C <sub>21</sub> H <sub>38</sub> O <sub>6</sub>	65.94	67.27	8.96	9.15	Yellow-orange	51.0
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	210-214/0 3	1 4858	1 0016	438.58	C <sub>25</sub> H <sub>42</sub> O <sub>6</sub>	68.45	68.04	9.65	9.89	Yellow-orange	50.7
Anisaldehyde	CH <sub>3</sub>	178-182/0 25	1 4855	1 1216	358.42	C <sub>19</sub> H <sub>20</sub> O <sub>7</sub>	60.31	60.26	8.44	8.27	Colorless	55.3
	C <sub>2</sub> H <sub>5</sub>	190-195/0 25	1 4805	1 0590	386.47	C <sub>21</sub> H <sub>28</sub> O <sub>7</sub>	62.15	62.42	8.87	8.96	Colorless	34.3
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	210-216/0 3	1 4748	1 0503	442.58	C <sub>25</sub> H <sub>42</sub> O <sub>7</sub>	65.13	65.08	9.57	10.07	Yellow	53.6
Cuminal	CH <sub>3</sub>	174-180/0 25	1 4788	1 0640	370.47	C <sub>20</sub> H <sub>32</sub> O <sub>6</sub>	64.84	64.71	9.25	9.35	Colorless	52.9
	C <sub>2</sub> H <sub>5</sub>	184-186/0 25	1 4738	1 0084	398.52	C <sub>22</sub> H <sub>38</sub> O <sub>6</sub>	66.30	66.09	9.61	9.77	Colorless	59.2
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	196-200/0.25	1 4720	0 9825	454.63	C <sub>26</sub> H <sub>46</sub> O <sub>6</sub>	68.68	68.66	10.20	10.03	Colorless	50.2
Piperonal	CH <sub>3</sub>	188-194/03	1 4918	1 2087	372.40	C <sub>19</sub> H <sub>20</sub> O <sub>8</sub>	58.05	58.25	7.58	7.58	Light yellow	42.9
	C <sub>2</sub> H <sub>5</sub>	196-200/0 3	1 4866	1 1193	400.46	C <sub>21</sub> H <sub>28</sub> O <sub>8</sub>	59.98	60.20	8.05	8.23	Light yellow	46.6
	<i>n</i> -C <sub>4</sub> H <sub>9</sub>	210-214/0 3	1 4805	1 0725	456.56	C <sub>25</sub> H <sub>40</sub> O <sub>8</sub>	63.13	63.12	8.83	8.57	Light yellow	50.0

toluene was added. The flask was then connected to a Dean-Stark distillation receiver and the contents gently heated to maintain boiling.

After twenty-four to thirty hours of refluxing, no further increase in water of reaction was observed. The reaction mixture was then allowed to cool and sodium methoxide added slowly, until a slightly basic reaction to litmus was observed. The mixture was then vacuum distilled, to leave only a tar-like aldehyde degradation product and a small residue of sodium methoxide. Repeated vacuum redistillations followed, until a constant refractive index was obtained, which served as an index of purity for the acetals.

The general reaction involved is:



The yields reported in Tables I and II refer to those obtained by using 0.1 M of aldehyde, 200 mg. of *p*-toluenesulfonylchloride, and a quantity of glycol ether sufficient to fulfill the molar ratio of 3 moles aldehyde to 5 moles glycol ether.

**Evaluation of the Glycol Ether Acetals for Aldehyde Release.**—Many of the acetals prepared retained moderate odors, similar to those of the parent aldehydes. *In vitro* evaluation of aldehyde release could be based on odor intensity comparisons between acetal alone, acetal in a slightly acidic medium, and pure aldehyde. Due to the subjectivity involved in odor intensity duplication by this method, it was felt that a chemical method would be more preferable.

The presence of free aldehyde was determined by reaction with Schiff's reagent (7). The modified Schiff's reagent of Carey (8) was employed. The usual pH of the intact skin remains a controversial aspect of epidermal physiology, with more than eleven investigations reporting values ranging from pH 3.0 to 7.0 (9, 10). It was therefore deemed advisable to average the reported experimental data and the preponderance of results placed the average pH of the human skin at about 5.5. Hence, a modified Schiff's test utilizing an aqueous medium buffered to pH 6.0 presented a medium equal to or of lesser acidity than the medium that the acetal would encounter upon application to the skin.

Test solutions of each glycol ether acetal and each aldehyde, for comparison as standards, were ethanol dilutions of such strength that a 2.0-ml. aliquot diluted to 25 ml. represented a  $1 \times 10^{-5}$  M solution with respect to acetal or aldehyde. The concentration of the glycol ethers under the same conditions was  $2 \times 10^{-5}$  M. Each glycol ether acetal was tested as follows: 5.0 ml. of pH 6.0 buffer (U. S. P. XV, page 931) was placed in each of three 25-ml. volumetric flasks; 2.0 ml. of acetal dilution was pipetted into the first flask; 2.0 ml. of the parent aldehyde dilution; and the corresponding volume of the appropriate glycol ether dilution were placed in the second flask. A blank consisting of 2.0 ml.

TABLE III—COLORIMETRIC ABSORBANCES OF ACETALS AND ALDEHYDE-GLYCOL-MONOETHERS AT 560 mμ USING THE MODIFIED SCHIFF'S REAGENT OF CAREY

Sample Composition	Acetal (I)	Aldehyde + Glycol mono-ether (II)	Absorbance Ratio (I/II)
<b><i>n</i>-Butyraldehyde</b>			
Ethylene glycol methyl ether	0.319	0.366	0.87
Ethylene glycol ethyl ether	0.366	0.398	0.91
Ethylene glycol <i>n</i> -butyl ether	0.387	0.420	0.92
Diethylene glycol methyl ether	0.356	0.377	0.94
Diethylene glycol ethyl ether	0.398	0.420	0.95
Diethylene glycol <i>n</i> -butyl ether	0.387	0.420	0.92
<b>Isodecaldehyde</b>			
Ethylene glycol methyl ether	0.371	0.398	0.93
Ethylene glycol ethyl ether	0.332	0.356	0.93
Ethylene glycol <i>n</i> -butyl ether	0.347	0.372	0.93
Diethylene glycol methyl ether	0.377	0.398	0.95
Diethylene glycol ethyl ether	0.342	0.366	0.93
Diethylene glycol <i>n</i> -butyl ether	0.337	0.377	0.89
<b>Benzaldehyde</b>			
Ethylene glycol methyl ether	0.155	0.210	0.74
Ethylene glycol ethyl ether	0.160	0.180	0.88
Ethylene glycol <i>n</i> -butyl ether	0.134	0.149	0.90
Diethylene glycol methyl ether	0.130	0.146	0.89
Diethylene glycol ethyl ether	0.160	0.180	0.88
Diethylene glycol <i>n</i> -butyl ether	0.130	0.150	0.81
<b>Cinnamaldehyde</b>			
Ethylene glycol methyl ether	0.418	0.456	0.91
Ethylene glycol ethyl ether	0.453	0.488	0.92
Ethylene glycol <i>n</i> -butyl ether	0.387	0.420	0.92
Diethylene glycol methyl ether	0.434	0.495	0.88
Diethylene glycol ethyl ether	0.515	0.553	0.93
Diethylene glycol <i>n</i> -butyl ether	0.545	0.585	0.93
<b>Anisaldehyde</b>			
Ethylene glycol methyl ether	0.097	0.142	0.68
Ethylene glycol ethyl ether	0.161	0.222	0.73
Ethylene glycol <i>n</i> -butyl ether	0.134	0.167	0.80
Diethylene glycol methyl ether	0.108	0.143	0.76
Diethylene glycol ethyl ether	0.167	0.215	0.77
Diethylene glycol <i>n</i> -butyl ether	0.132	0.167	0.79
<b>Cuminal</b>			
Ethylene glycol methyl ether	0.387	0.398	0.97
Ethylene glycol ethyl ether	0.319	0.372	0.86
Ethylene glycol <i>n</i> -butyl ether	0.352	0.387	0.91
Diethylene glycol methyl ether	0.314	0.366	0.86
Diethylene glycol ethyl ether	0.319	0.398	0.80
Diethylene glycol <i>n</i> -butyl ether	0.334	0.366	0.91
<b>Piperonal</b>			
Ethylene glycol methyl ether	0.347	0.351	0.99
Ethylene glycol ethyl ether	0.337	0.366	0.92
Ethylene glycol <i>n</i> -butyl ether	0.347	0.347	1.00
Diethylene glycol methyl ether	0.361	0.377	0.96
Diethylene glycol ethyl ether	0.342	0.366	0.93
Diethylene glycol <i>n</i> -butyl ether	0.337	0.351	0.87

ethanol and 20 ml glycol ether dilution was prepared in the third flask. Exactly ten minutes standing time at room temperature was allowed for the hydrolysis reaction. Then 10 ml Carey's reagent and sufficient distilled water were added to bring the volume to exactly 250 ml. The addition of 20 ml ethanol to the solutions containing acetals of ethylene glycol monobutyl ether was necessary to insure complete solubility. The solutions were allowed to stand for an additional ten-minute period at room temperature to assure complete color development. The color produced in the aldehyde and acetal dilutions was then measured at 560 m $\mu$  relative to the reagent blank adjusted to 100% transmission. The results obtained are summarized in Table III.

### DISCUSSION

With the exception of the anisaldehyde acetals, the absorbances of the hydrolyzed acetal samples were almost equivalent to those of the corresponding aldehyde dilutions. The behavior of the anisaldehyde

acetal may be explained by the strong electron-donating effect of the methoxy group which is transmitted through the benzene ring to the bond in the Schiff's base linkage. It may therefore be concluded that under the specified experimental conditions aqueous solutions buffered to pH 6.0 were capable of hydrolyzing these acetals to the parent aldehydes.

### REFERENCES

- (1) Takada, S., *Kogyo*, **24**, 10(1953)
- (2) Betzler, E., *Ather, Ole, Riechstoffe, Parfumer, Essen: Aromen*, **1**, 82(1951)
- (3) Nieuwland, J., U S pat 1,824,963, Sept 29, 1931
- (4) MacDowell, G., and McNamee, R. W., U S pat 2,321,094, June 8, 1943
- (5) Neurath, M., *Am Perfumer Essent Oil Ret*, **61**, 273(1953)
- (6) Kulka, M., *ibid*, **54**, 136(1949)
- (7) Snell, F. D., and Snell, C. T., "Colorimetric Method of Analysis," 3rd Ed, Vol II, D. Van Nostrand Co, New York, N. Y., 1953, p. 259
- (8) Carey, M. W., Green, L. W., and Schoetzw, R. E. *THIS JOURNAL*, **22**, 1237(1933)
- (9) Jacobi, O., and Heinrich, H., *Proc Sci Sect Toile Goods Assoc*, **21**, 6(1954)
- (10) Lubowe, I., *ibid*, **23**, 40(1955).

## Acid Hydrolysis of Tetrahydropyranyl Glycosides: Effects of 2-Methylation and 3-Hydroxylation on Rate of Hydrolysis\*

By ROBERT V. PETERSEN

A series of methyl glycosides (acetals and ketals) analogous to aldopyranosides, ketopyranosides, and their 3-hydroxy analogs (tetrahydropyran, 2-methyl-tetrahydropyran, and 3-hydroxyl derivatives of these compounds) were synthesized, subjected to acids of various concentrations, and their rates of hydrolysis determined spectrophotometrically.

A CHARACTERISTIC feature of glycosides is the bonding of a sugar to an aglycon by an acetal or ketal bond. This type of bonding is known to be relatively unstable in the presence of acids, the degree of instability being dependent on the chemical nature of the sugar and the aglycon. Because orally ingested glycosides are immediately subjected to the relatively strongly

acidic conditions in the stomach, a knowledge of the behavior of various fundamental glycoside under such conditions might suggest the synthesis of new agents which possess more desirable stability, as well as absorption and distribution characteristics.

Several factors serve to regulate the stability of acetals and ketals to acid hydrolysis. For example, it has already been demonstrated that glycosides in which the 3-position of the sugar moiety contains a hydroxyl group are much more stable than the corresponding 3-desoxy glycosides (1, 2). Similarly, glycosides of aldehyde-sugars are more stable than corresponding glycosides of keto-sugars (3). It must also be realized that other factors found in naturally occurring glycosides, such as the nature of the alcohol, the presence of additional hydroxyl groups in the sugar,  $\alpha$ - and  $\beta$ -forms of the glycoside, and other variations also influence the stability of the compounds.

\* Received January 25, 1960, from the University of Utah, College of Pharmacy, Salt Lake City 1.

This investigation was supported in part by a grant from the University of Utah Research Fund.

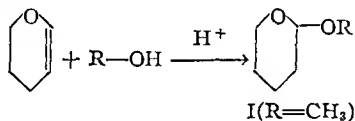
Presented to the Scientific Section, A PH A Cincinnati meeting, August 1959.

The author is grateful to Mr. Glenn Rollson for his assistance with some of the spectrophotometric determinations.

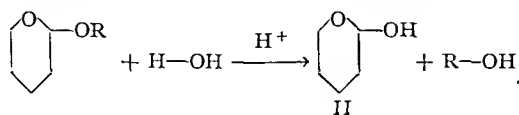
There are conflicts in the literature regarding the stability of some acetals and ketals. For example, Bergmann and Mickleley (4, 5), using chemical methods of assay, state that 2-methyl-2-methoxy-tetrahydropyran is quantitatively hydrolyzed within a few minutes in 0.0005*N* hydrochloric acid, whereas Linnell and Melhuish (6), using chemical and enzymatic methods, report that this compound is resistant to hydrolysis by 1 *N* hydrochloric acid during seventy-two hours, and is stable to permanganate, and, hence, is comparable in reactivity to the normal sugars. Linnell and Melhuish also claim this to be a new substance, although it had been reported previously by Bergmann and Mickleley.

In view of the importance of glycosides as drugs, and of the divergence of opinions relative to the stability of the acetal and ketal bond, it appeared important to study more definitively, with newer techniques, the relative stability of a series of acetals and ketals, with special reference to the effects of 2-methylation and 3-hydroxylation of these compounds. The results of numerous experiments performed provide the basis for this report.

Cyclic vinyl ethers, such as dihydropyran, are highly reactive to a variety of reagents, such as alcohols. Paul (7) showed that dihydropyran reacts rapidly with hydroxyl-containing compounds, in the presence of an acid catalyst, to yield acetals.

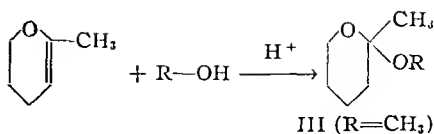


In the presence of aqueous acids, the acetal is hydrolyzed to 2-hydroxy-tetrahydropyran (II) ( $\delta$ -hydroxyvaleraldehyde) and the alcohol.

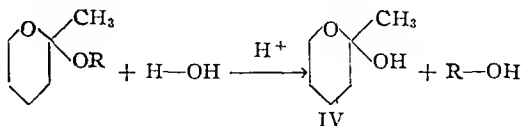


Parham and Anderson (8) suggested that this reaction be used advantageously as a means of protecting the hydroxyl group during reactions carried out in an alkaline medium, as the acetal is relatively stable in alkaline solutions and can be restored to the original hydroxy-compound by mild acid hydrolysis.

Ketals of 2-methyl- $\Delta^2$ -dihydropyran (anhydro- $\gamma$ -aceto-*n*-butyl alcohol) are formed by reaction of 2-methyl- $\Delta^2$ -dihydropyran with an alcohol (4, 5).



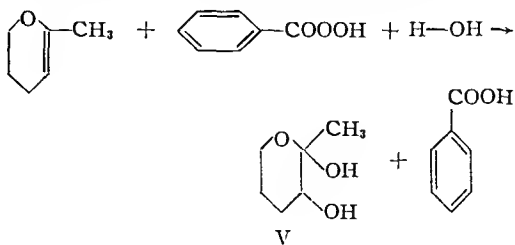
Acid hydrolysis of this ketal yields 2-methyl-2-hydroxy-tetrahydropyran (IV) ( $\gamma$ -aceto-*n*-butyl alcohol) and the alcohol (7, 9-11).



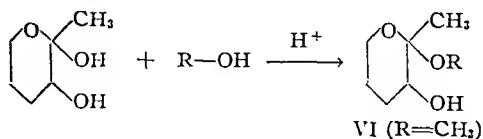
It has been shown by spectroanalysis that  $\delta$ -hydroxy aldehydes, such as  $\delta$ -hydroxy-valeraldehyde, exist primarily in the cyclic form as hemiacetals (11-14), whereas the  $\delta$ -hydroxy ketone,  $\gamma$ -aceto-*n*-butyl alcohol (IV) has been shown to exist mainly in the open chain form (14). The reactions of both types of compounds are explained mainly on the basis of the cyclic hemiacetal and hemiketal structures. This is evidenced by the fact that each type of compound will react with alcohols to form cyclic acetals and ketals which are identical to those formed from analogous cyclic vinyl ethers and alcohols (4-6).

Naturally occurring glycosides, likewise, are typified by the acetal linkage of hydroxyl-containing compounds to various sugars. The analogy between compounds II and IV with sugars and between I and III with glycosides was pointed out by Bergmann and Mickleley (4, 5) and by Linnell and Melhuish (6).

3-Hydroxy- $\gamma$ -aceto-*n*-butyl alcohol was prepared by Bergmann and Mickleley (4) by hydroxylation of anhydroacetobutyl alcohol with perbenzoic acid, and was shown to exist in equilibrium with the pyranose hemiacetal form, 2-methyl-2,3-dihydroxy-tetrahydropyran (V).

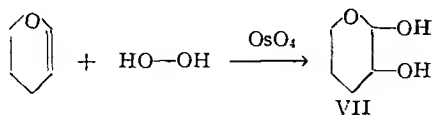


Compound V underwent condensation with alcohols, similar to the reactions of acetobutyl alcohol, to yield cyclic ketals.



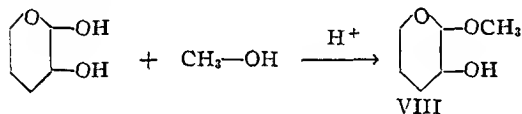
Compound V and its methyl acetal VI were compared with other carbohydrates and glycosides and analogies were made in their properties and reactivities.

Recently, Hurd and Kelso (15), and Hurd, Moffatt, and Rosnati (16) prepared 2,3-dihydroxy-tetrahydropyran (VII) from dihydropyran and hydrogen peroxide in the presence of osmium tetroxide.

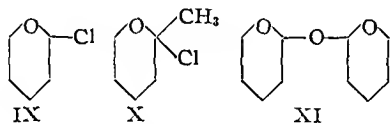


The resulting carbohydrate was compared with other desoxyaldopentoses (15, 17).

In order to complete the series of acetals and ketals to be studied, the methyl acetal of compound VII was synthesized by reacting VII with 1% methanolic hydrochloric acid. The resulting compound was 2-methoxy-3-hydroxy-tetrahydropyran (VIII).



In addition to the above compounds, 2-chloro-tetrahydropyran (IX), 2-chloro-2-methyl-tetrahydropyran (X), and the "disaccharide" 2'-tetrahydropyran-2-yl-2-tetrahydropyran (XI) were synthesized and tested for stability in dilute aqueous acids.



## EXPERIMENTAL

The physical properties and references to the syntheses of the various compounds are listed in Table I.

**Synthesis of 2-Methoxy-tetrahydropyran (I).**—Compound I was synthesized by the method of Paul (7) as modified by Woods and Kramer (11). The synthesis involves the reaction of dihydropyran with methanol containing hydrochloric acid, neutralization of the acid with sodium hydroxide pellets, then distillation of the product.

**Synthesis of 2-Hydroxy-tetrahydropyran (II).**—Dihydropyran, purified by drying over Drierite<sup>1</sup> for several days, then fractionating at 83.8–84.0° at 742 mm., was hydrated by the method of Schniepp and Geller (18), using aqueous hydrochloric acid, to yield II. Methylation of II in 1% methanolic hydrochloric acid at 50° for one hour and fractionation of the mixture yielded a product identical with I.

**Synthesis of 2-Methyl-2-hydroxy-tetrahydropyran (IV) (γ-Aceto-*n*-butyl Alcohol).**—Compound IV and its anhydro-derivative, 2-methyl-Δ<sup>2</sup>-dihydropyran (anhydro-γ-aceto-*n*-butyl alcohol) were prepared by the method of Lipp (19, 20), with modifications (6, 21). The synthesis involves the mono condensation of 1,3-dibromopropane with the sodium salt of ethylacetoacetate, followed by acid hydrolysis to IV. The anhydro- derivative formed by gradual heating of IV to a temperature below its boiling point, at which point water is lost from the molecule and the anhydro- product distills, along with the water.

**Synthesis of 2-Methoxy-2-methyl-tetrahydropyran (III).**—Compound III was synthesized by mixing IV with 1% methanolic hydrochloric acid, heating the mixture to 50° for one hour, and fractionating the mixture without neutralization. The product distilled at 131–133°, which does not agree well with that reported to be at 116–117° (6). An identical product was obtained when the anhydro- derivative, 2-methyl-Δ<sup>2</sup>-dihydropyran, was allowed to react with methanol containing a trace of hydrochloric acid, the reaction being more vigorous and highly exothermic.

**Synthesis of 2-Methyl-2,3-dihydroxy-tetrahydropyran (V).**—Compound V was prepared from 2-methyl-Δ<sup>2</sup>-dihydropyran by hydroxylation with an ethereal solution of perbenzoic acid, saturated with water, by the method of Bergmann and Mieleke (5). The perbenzoic acid was prepared from benzoyl peroxide and sodium methoxide in chloroform by the method of Tiffeneau (22). The resulting compound was a crystalline solid.

**Synthesis of 2-Methoxy-2-methyl-3-hydroxy-tetrahydropyran (VI).**—This synthesis was carried out in a manner similar to that reported by Bergmann and Mieleke (4, 5). Compound V, dissolved in 0.01 *N* methanolic hydrochloric acid, was allowed to react for one hour at room temperature, after which the mixture was fractionated, yielding VI.

**Synthesis of 2,3-Dihydroxy-tetrahydropyran (VII).**—Compound VII was synthesized by the osmium tetroxide catalyzed hydroxylation of purified dihydropyran by the method of Hurd and Kelso (15).

**Synthesis of 2-Methoxy-3-hydroxy-tetrahydropyran (VIII).**—Ten grams of VII was dissolved in 50 ml. of 1% methanolic hydrochloric acid and the resulting solution was allowed to stand at room temperature for one hour, after which it was heated to 50° for one hour. Fractionation of the resulting mixture yielded a liquid which boiled at 48–53°. It was clear, colorless, and very mobile in contrast to the syrupy VII. Compound VIII had a faint pleasant odor.

*Anal.*—Calcd. for C<sub>6</sub>H<sub>12</sub>O<sub>3</sub>: C, 54.5; H, 9.0; Found: C, 53.6; H, 9.08.

**Synthesis of 2-Chloro-tetrahydropyran (IX).**—Dry hydrogen chloride gas was bubbled into 8.4 Gm (0.1 mole) of purified dihydropyran until 3.65 Gm (0.1 mole) had been absorbed (23). External cooling was applied during the addition of hydrogen chloride. The product was distilled several times under reduced pressure, yielding IX.

**Synthesis of 2-Chloro-2-methyl-tetrahydropyran (X).**—Compound X was prepared in a manner analogous to the synthesis of IX. Dry hydrogen chloride gas was bubbled through 9.8 Gm. (0.1 mole)

<sup>1</sup> W. A. Hammond Drierite Co.

TABLE 1—PHYSICAL PROPERTIES AND SYNTHESIS REFERENCES OF THE VARIOUS COMPOUNDS

Com- pound	Synthesis Reference	Boiling Point, °C		n <sub>D</sub> <sup>b</sup>		Absorption Maximum, mμ
		Reported	Found	Reported	Found	
I	(7, 11)	125 (7) 128 (11)	123-125	1 4262	1 4257	270
II	(18)	54-55/3 mm	54-56/3 mm	1 4514 <sup>c</sup>	1 4461 <sup>c</sup>	Beyond 220
III	(4-6)	76/100 mm (4, 5) 116-117 (6)	78-80/100 mm 131-133	1 4273	1 4278	Beyond 220
IV	(4-6, 19, 20)	110-115/22 mm (6)	109-115/20 mm.	1 4438 <sup>d</sup>	1 4422 <sup>d</sup>	270
V	(4)	<sup>e</sup>	<sup>f</sup>			277
VI	(4)	<sup>g</sup> 76-77/9-10 mm.	78-79/12 mm	1 4542 <sup>g</sup>	1 4538 <sup>g</sup>	Beyond 220
VII	(15, 16)	<sup>h</sup>	<sup>h</sup>		1 4809	266
VIII			52-53/1-2 mm		1 4533	Beyond 220
IX	(23)	42/11 mm	49-50/20 mm			Beyond 220
X			45-48/18 mm			235 (water) Beyond 223 (cyclo- hexane)
XI	(7)	106-110/12 mm	45-50/1 mm	1 46589 <sup>i</sup>	1 4638 <sup>i</sup>	270 (water) Beyond 223 (cyclo- hexane)

<sup>a</sup> Corrected to atmospheric pressure unless otherwise noted <sup>b</sup> Taken at 20° unless otherwise noted <sup>c</sup> Taken at 25°  
<sup>d</sup> Taken at 21° <sup>e</sup> Melting point, 73° <sup>f</sup> Melting point, 72-73° <sup>g</sup> Taken at 18° <sup>h</sup> Heavy syrup, purified by distillation  
as the diacetate at 109-111°/1-2 mm, then deacetylated <sup>i</sup> Taken at 17°

of 2-methyl-Δ<sup>2</sup>-dihydropyran until 3.65 Gm (0.1 mole) had been absorbed. The product distilling at 40-50° at 18 mm was collected and redistilled at 45-48° at 18 mm.

Compounds IX and X were very unstable, even at 0° Fuming, discoloration, and polymerization were noted after standing for a few days. The rates of hydrolysis of these compounds were determined immediately after distillation. The 2-bromo-derivative of 2-methyl-tetrahydropyran had been previously synthesized, but was too unstable to be isolated in pure form (10).

**Synthesis of 2'-Tetrahydropyranloxy-2-tetrahydropyran (XI).**—Compound XI was synthesized by mixing dihydropyran with II and catalyzing the reaction by the addition of hydrochloric acid, by the method of Paul (7). An identical product was obtained from a mixture of IX and II, or by dehydration of II.

**Anal.**—Calcd for C<sub>10</sub>H<sub>18</sub>O<sub>3</sub>: C, 64.5; H, 9.67. Found: C, 64.1; H, 9.64.

**Spectrophotometric Analysis of Rates of Hydrolysis.**—All analyses were run in a Beckman model DU spectrophotometer, equipped with a Nylab voltage stabilizer connected with a 115 volt a-c power supply through a Sol constant voltage stabilizer. The temperature within the cell compartment of the spectrophotometer was maintained at 29-30° by water cooling of the lamp housing. All readings were made between 220 and 340 mμ, thus a hydrogen lamp was used as the light source throughout all of the experiments.

The absorption maximum was determined for each compound analyzed (see Table I) either in water, dilute acids, or an anhydrous solvent, such as cyclohexane. Acid concentrations were carefully standardized. The more dilute acids were prepared by dilution of the more concentrated standard acids. All water used was deionized, distilled, and freshly boiled. The cyclohexane used was Spectro grade.<sup>2</sup>

**Procedure.**—After establishment of the absorption maximum for each compound, the rates of hydrolysis of the glycosides and chloro-compounds

were determined in acids of various concentrations. In following the rates of hydrolysis of the glycosides to their respective sugars plus methanol, i.e., I → II + methanol, III → IV + methanol, VI → V + methanol, VIII → VII + methanol, XI → 2II, and of the chloro-compounds to their respective sugars plus hydrochloric acid, i.e., IX → II + hydrochloric acid, X → IV + hydrochloric acid, each compound being hydrolyzed was run against the solvent as the blank. Simultaneously, equimolar concentrations of the sugar plus methanol, where applicable, were run against the same solvent. The rates at which the absorbances of the hydrolyzing solutions approached that of the sugar solutions were determined. The rates of hydrolysis were determined either by comparison with absorbances of solutions of known composition, or by calculation.<sup>3</sup> For example, a mixture containing III, IV, and methanol, each in a 0.01 M concentration, corresponds to 50% hydrolysis of a 0.02 M solution of III. Other points corresponding to 25, 75, and 90% hydrolysis were similarly determined for each hydrolyzing compound, where possible. Only the 50% hydrolysis, corresponding to the half-lives of the compounds, are herein reported (see Table II). Unless otherwise noted, 0.02 M concentrations of glycoside, sugar, and methanol were employed.

The hydrolysis of I to II was determined at 224 mμ. This does not correspond to the maximum for either I or II. However, it was found that an intermediate compound apparently forms during hydrolysis, which has its maximum at that point.

The hydrolysis of III to IV was determined at 270 mμ, the absorption maximum of IV. The absorption maximum of III was beyond 220 mμ. The hydrolysis of VI to V was determined at 277 mμ, the absorption maximum of V. The maximum of VI was beyond the 220 mμ range. The hydrolysis of VIII to VII was determined at 266 mμ, the absorption maximum of VII in acids.

The rate of hydrolysis of IX to II was determined at 235 mμ, the absorption maximum of IX in water.

<sup>3</sup> The author is indebted to Dr. Richard P. Smith for assistance with some of the calculations.

<sup>2</sup> Eastman Kodak Co.



TABLE II — HALF LIVES<sup>a</sup> OF THE VARIOUS GLYCOSIDES AND CHLORO COMPOUNDS IN HYDROCHLORIC ACID OF VARIOUS CONCENTRATIONS AND pH 7 BUFFER SOLUTION

Compound	Acid Concentration						pH7 Buffer
	1 N	0.1 N	0.01 N	0.001 N	0.0001 N	0.00001 N	
I	2.5	100	1,200				
III				<0.2	0.2	8	102
VI		<0.25	0.78	6	240	2,400	
VIII	> hours						
IX	Very rapid hydrolysis in all aqueous solvents						
X	Very rapid hydrolysis in all aqueous solvents						
XI	2	60					

<sup>a</sup> Figures indicate time in minutes

In cyclohexane, the maximum was beyond 223 mμ. The rate of hydrolysis of X to IV was determined at 270 mμ, the absorption maximum of IV in water. Compound X exhibited a maximum beyond 223 mμ in cyclohexane.

The rate of hydrolysis of XI to II was determined at 224 mμ. During the hydrolysis, the absorption maximum of XI shifted from 270 mμ to that point. A 0.01 M solution was used, with the expectation that after hydrolysis the absorption would correspond to that of a 0.02 M solution of II.

RESULTS

The results of acid hydrolysis of the various glycosides and the disaccharide (XI) are shown graphically in Figs 1-5. The half-lives of the hydrolyzing solutions are given numerically in Table II.

The results of the hydrolysis of IX indicated that it was almost immediately hydrolyzed by water or acids. However, the absorption maximum for this compound in water was noted to be 235 mμ, which does not correspond with the absorption of II, as would be expected. It was synthesized in the belief that it might be the intermediate formed during the hydrolysis of I. However, as the absorption does not correspond with that of the intermediate (224 mμ), this appears doubtful. No explanation is offered at this time for its behavior. The absorption maximum for this compound in cyclohexane is beyond 223 mμ.

The hydrolysis of X was measured at 270 mμ, the absorption maximum of IV. All measurements, in water or acids, indicated complete hydrolysis of compound X within fifteen seconds, the time required to

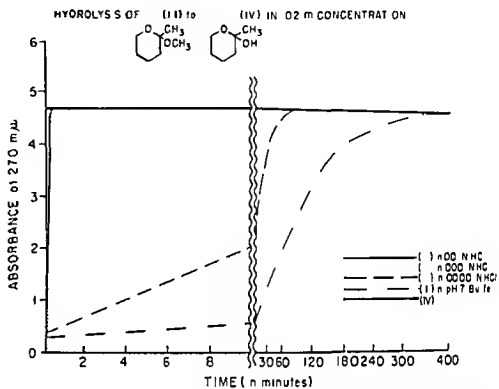


Fig 2 — Hydrolysis of III to IV in 0.001 N, 0.0001 N, 0.00001 N HCl, and in pH 7 buffer

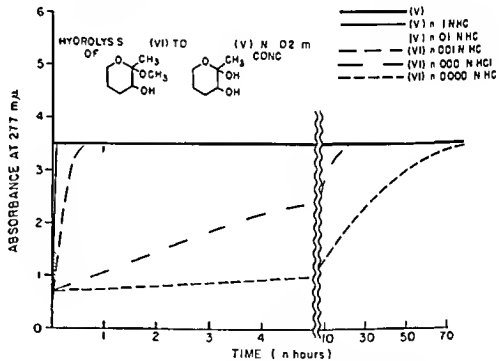


Fig 3 — Hydrolysis of VI to V in 0.1 N, 0.01 N, 0.001 N, 0.0001 N, and 0.00001 N HCl

make the first reading after dissolving the compound in the solvent. Absorbance measurements agreed well with those of solutions of IV in equimolar concentrations. When examined in cyclohexane, X exhibited an absorption maximum beyond 223 mμ.

DISCUSSION

From Fig 1 it can be seen that during the hydrolysis of I an intermediate compound of unknown identity, is formed. A question may arise as to whether this intermediate is formed before, during, or after hydrolysis occurs. Figure 5 indicates that it occurs during hydrolysis, as compound XI hydrolyzes to form an intermediate having an absorption maximum at the same wavelength. It can also be seen from Fig 5 that from a 0.01 M solution

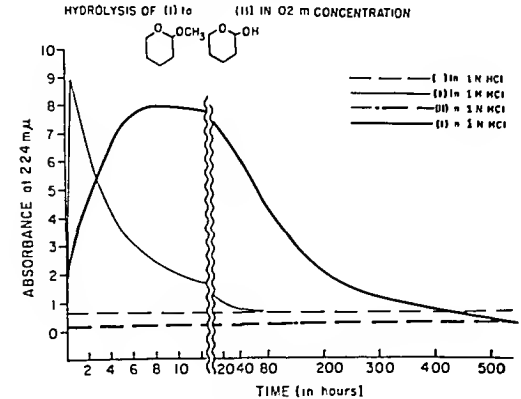


Fig 1 — Hydrolysis of I to II in 0.1 N and 1 N HCl

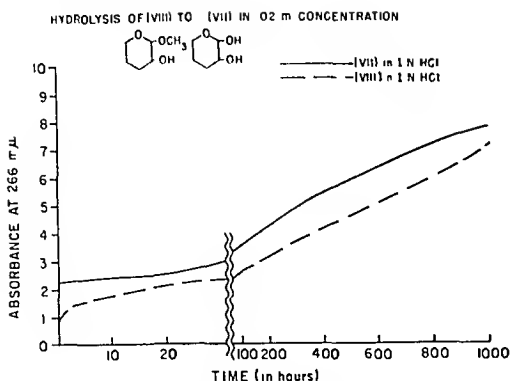


Fig 4.—Hydrolysis of VIII to VII in 1 N HCl

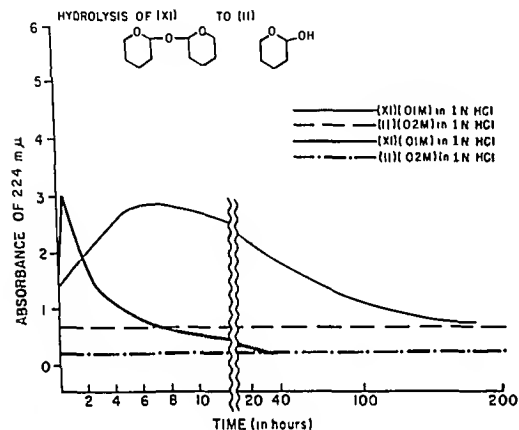


Fig 5.—Hydrolysis of XI to II in 1 N and 0.1 N HCl.

of XI, eventually a 0.02 M solution of II is formed. However, during the hydrolysis, the absorption does not reach the point attained by the hydrolysis of a 0.02 M solution of I. Apparently the intermediate forms from only one-half of the disaccharide XI. It will also be noted that the methyl glycoside I hydrolyzed at approximately the same rate as did the disaccharide XI in acids of the same concentration.

From Fig 3 it can be seen that both VII and VIII are affected by 1 N hydrochloric acid, the concentration of acid required to effect hydrolysis of VIII.

The rates of hydrolysis of compounds III and VI were measured graphically by plotting against the transmittance of solutions of known concentrations containing the glycosides and their hydrolytic products, the sugars, and methanol.

From the results tabulated it can be seen that there is a several thousandfold difference in the rates of hydrolysis of compounds I and III as a result of 2-methylation. A similar comparison between VI and VIII substantiates this observation. The differences attributable to 3-hydroxylation may be seen by comparing the half-lives of compounds I and VIII, or of compounds III and VI. The data thus obtained are in agreement with previous reports (1-3, 24) relating to the increased rate of hydrolysis of glycosides due to 2-methylation, and the de-

creased rate due to 3-hydroxylation, the greater effect being attributable to 2-methylation.

The results of the hydrolysis of compound III, as shown in Fig 2 and in Table II, are in agreement with the data reported by Bergmann and Miekeley (4, 5), and disagree markedly with that reported by Linnell and Melhuish (6).

## SUMMARY

1. 2-Methoxy-tetrahydropyran, 2-methoxy-2-methyl-tetrahydropyran, 2-methoxy-3-hydroxy-tetrahydropyran, 2-methoxy-2-methyl-3-hydroxy-tetrahydropyran, 2-chloro-tetrahydropyran, 2-chloro-2-methyl-tetrahydropyran, and 2'-tetrahydropyranyloxy-2-tetrahydropyran were synthesized and subjected to acids of various concentrations and their rates of hydrolysis determined by following the changes in absorbance.

2. The free hemiacetals of each of the above compounds were synthesized. Solutions of these compounds were examined spectrophotometrically, their absorbances being used as the basis for determining the end point of the hydrolysis of the above listed compounds.

3. Studies indicate that 2-methyl substitution in tetrahydropyran increases the rate of hydrolysis of the methyl glycoside several thousand times.

4. Studies indicate that 3-hydroxylation of the methyl glycosides of tetrahydropyran derivatives decreases the rate of hydrolysis in the range of 200 to 1,200 times, depending somewhat on pH.

## REFERENCES

- (1) Bergmann, M., Schotte, H., and Lechinsky, W., *Ber*, 55, 158(1922).
- (2) Pigman, W. W., and Goepf, R. M., Jr., "Chemistry of the Carbohydrates," Academic Press Inc., New York, N. Y., 1948, pp 203-207.
- (3) Ruber, C. N., and Sorensen, N. A., *Det Kgl Norske Videnskabs Selskabs Skrifter*, 1938, 1.
- (4) Bergmann, M., and Miekeley, A., *Ann*, 432, 319 (1923).
- (5) Bergmann, M., and Miekeley, A., *Ber*, 55, 1390 (1922).
- (6) Linnell, W. H., and Melhuish, B. W., *Quart J Pharm and Pharmacol*, 3, 40(1930).
- (7) Paul, R., *Bull soc chim France*, 1, 973(1934).
- (8) Parham, W. E., and Anderson, E. L., *J Am Chem Soc*, 70, 4184(1948).
- (9) Paul, R., *Bull soc chim France*, 4, 53, 1489(1933).
- (10) Paul, R., *ibid*, 5, 2, 311, 2200(1935).
- (11) Woods, G. F., and Kramer, D. N., *J. Am Chem Soc*, 69, 2246(1947).
- (12) Helfferich, B., "Advances in Carbohydrate Chemistry," Academic Press, Inc., New York, N. Y., 1952, pp. 209-245.
- (13) Helfferich, B., *Ber*, 52B, 1800(1919).
- (14) Auwers, K. V., *ibid*, 56B, 1672(1923).
- (15) Hurd, C. D., and Kelso, C. D., *J Am. Chem. Soc.*, 70, 1484(1948).
- (16) Hurd, C. D., Moffatt, J., and Rosnati, L., *ibid*, 77, 2793(1955).
- (17) Hurd, C. D., and Edwards, D. E., *J Org Chem*, 14, 680(1949).
- (18) Schniepp, L. E., and Geller, H. H., *J Am Chem Soc*, 68, 1646(1946).
- (19) Lipp, A., *Ann*, 289, 181(1896).
- (20) Lipp, A., *Ber*, 18, 3275(1885).
- (21) Petersen, R. V., and Gisvold, O., *THIS JOURNAL*, 45, 572(1956).
- (22) Trifeneau, M., "Organic Syntheses," Coll Vol 1a, John Wiley & Sons, Inc., New York, N. Y. 1941, pp 422.
- (23) Publication 823, E. I. du Pont de Nemours and Co., Inc., Niagara Falls, N. Y.
- (24) Isbell, H. S., and Frush, H. L., *J Research N B Bur Standards*, 24, 125(1940).

# Chromatographic Separation of the Phenolic Compounds of *Cannabis sativa*\*

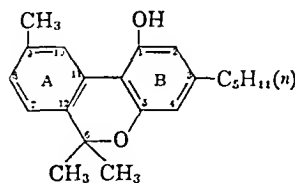
By ROBERT S. DE ROPP

A method is given for partially purifying the phenolic components of *Cannabis sativa* resin and for separating these components by means of paper chromatography. The application of this method for the preparation of pure cannabinal and tetrahydrocannabinol is described.

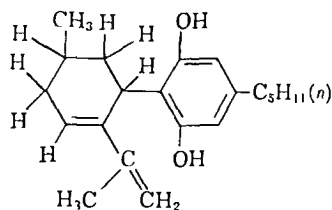
THE ACTIVE principle of the resin from *Cannabis sativa*, commonly known as red oil of hemp, resisted for many years the efforts of chemists to purify it and determine its structure. It was not until 1941 that Wollner, Matchett, Levine, and Loewe (1) described the isolation from Indian charas of tetrahydrocannabinol which was very potent physiologically, as manifested by its effect on dogs. This material, a colorless, viscous, optically active oil, was isolated as the acetate.

Adams and co-workers had already, at this time, determined the structure of cannabinal (2), isolated cannabidiol (3) and isomerized it to two isomeric, physiologically active tetrahydrocannabinols (4). Subsequently Adams and co-workers synthesized a series of analogs of tetrahydrocannabinol. One of the compounds they made had a potency considerably greater than that of natural tetrahydrocannabinol.

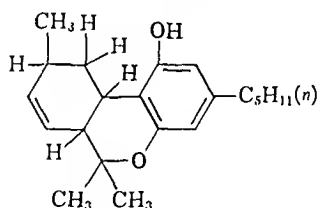
These compounds belong in a class by themselves, being completely unlike any other agents affecting the central nervous system. Chemically, they are all dibenzopyrans, their pharmacological activity being affected by the nature of the substituents and the location of double bonds. Some, like the tetrahydrocannabinols, are viscous, colorless oils. Others, (cannabinal and cannabidiol) can be crystallized. The chemical structures of some of the naturally occurring and synthetic substances belonging to this group are shown below.



I Cannabinal



II Cannabidiol



III Tetrahydrocannabinol

Stereo and optical isomerism make possible the existence of several different tetrahydrocannabinols. Of the synthetic compounds assayed by the dog ataxia test, potency was shown to be affected by the length of the side chain in position 3, the substituent  $-\text{CH}(\text{CH}_3)\text{C}_7\text{H}_{15}$  conferring a potency 32.6 times that of the  $\text{C}_5\text{H}_{11}(n)$  standard and about twice that of natural tetrahydrocannabinol acetate (5).

Despite the recent upsurge of interest in chemopsychiatric agents little work has been done on the mode of action of tetrahydrocannabinol. It produces in man a variety of reactions ranging from euphoria to depression, but the extreme unpredictability of its action has discouraged its use though synthetic preparations of the resin (Synhexyl or Pyrahexyl) have been available for several years. The pharmacology of these substances has been reviewed by Locwe (6).

## EXPERIMENTAL

**Preparation of Phenolic Fraction.**—Forty pounds of Mexican marihuana<sup>1</sup> was reduced to a coarse powder in a mill. The powdered material was processed in batches of about 6 Kg., each batch stirred with 6 L. of methanol, packed in a 12 × 155-cm. column, and allowed to stand for twenty-four hours. The mass was percolated with methanol for a further twenty-four hours, 12 L. of percolate

\* Received January 15, 1960, from the Biochemistry Department, Biochemical Research Section, Lederle Laboratories, A Division of American Cyanamid Co., Pearl River N. Y.

<sup>1</sup> The author is indebted to Mr. H. J. Anslinger, Commissioner of Narcotics, and Dr. Nathan B. Eddy of the National Institutes of Health for making this material available.

being recovered. The solvent was removed *in vacuo* and the residue was suspended in 4 L. of water which was extracted three times with 600 ml. of petroleum ether. Solvent was removed from the petroleum ether fraction which yielded 212.22 Gm. of solids. The aqueous fraction was discarded.

Solids from the petroleum ether fraction were dissolved in fresh petroleum ether and passed through a 90 × 600-mm. column of 1,720 Gm. Florisil (activated magnesium silicate) 100-200 mesh. The column was eluted with a further 2 L. of petroleum ether. The first 600 ml. containing a colorless oil was rejected. The second fraction containing a red oil was collected and freed from solvent. The yield was 10.286 Gm. of red oil. This red oil fraction produced ataxia in a dog at 25 mg./Kg.<sup>2</sup>

Colored impurities were removed from the red oil fraction by passing the material in petroleum ether through a second 40 × 600-mm. column packed with 160 Gm. Florisil, eluting with 500 ml. petroleum ether which removed an inert oil, followed by 750 ml. benzene which removed 1.123 Gm. of a mixture of phenolic compounds in the form of an almost colorless resin (referred to as the phenolic fraction). In other preparations of red oil, large amounts of inert oil from the hemp seeds first had to be removed by passing the material in chloroform through a column of alumina. The inert oil was eluted by chloroform; the phenolic fraction was removed with methanol.

**Paper Chromatography of the Phenolic Fraction.**—As the phenolic fraction was almost completely insoluble in water, a nonaqueous solvent system had to be found capable of separating its components. The system, cyclohexane:N,N-dimethylformamide, 10:1, gave satisfactory results. Sheets of Whatman No. 1 paper were first spotted with the material to be chromatographed. The sheets were then passed quickly through a bath containing the dimethylformamide phase of the above system until the line of solvent was about 1 cm. below the starting line. The sheets were then blotted, placed in the chromatography chamber, and left for one hour to come to equilibrium with the solvent vapors in the jar. The mobile phase (cyclohexane) was then added and the strips allowed to develop (descending) for eight hours. After drying, the sheets were examined in the light transmitted from a germicidal lamp passed through filter No. 9863 (Corning). Absorbing and fluorescent spots were marked. The sheets were then sprayed with freshly made diazotized sulfanilic acid, with which reagent the phenolic components of the material gave yellow or orange chromophores.

Table I shows the results obtained by means of this type of chromatography.

Eight spots developed on the strips after spraying with diazotized sulfanilic acid. Three of these ( $R_f$  0.22, 0.36, 0.55) absorbed in the U. V. sufficiently strongly to be visible when the sheet was laid over a U. V. source. Both the color and the rate at which it developed with diazotized sulfanilic acid varied with different compounds. Crystalline cannabidiol produced a lemon-yellow spot almost instantly which had an  $R_f$  value of 0.12 and corresponded to spot No. 2 in the phenolic fraction. Cannabinol<sup>3</sup>

TABLE I.— $R_f$  VALUES, U. V. ABSORPTION, AND REACTIONS TO DIAZOTIZED SULFANILIC ACID (DSA) OF COMPONENTS OF THE PHENOLIC FRACTION OF *Cannabis sativa*

$R_f$ of Spot	Intensity of U. V. Absorption	Color With DSA	Probable Identity
0.07	—	Pale yellow	?
0.12	—	Pale yellow	Cannabidiol
0.22	+	Brown	?
0.36	++++	Orange	Cannabinol
0.41	—	Yellow	?
0.55	+++	Yellow	Tetrahydrocannabinol
0.61	—	Yellow	?
0.73	—	Yellow	?
0.93	—	Yellow	?

gave an orange spot which did not become visible until thirty minutes after spraying. It corresponded to spot No. 4 ( $R_f$  0.36) in the phenolic fraction and was the spot which absorbed most strongly in the U. V. spot No. 5 in the phenolic fraction overlapped with No. 4 and gave a yellow color which developed more rapidly than did the orange spot of cannabinol. Spot No. 6 ( $R_f$  0.55) corresponded to the pharmacologically active fraction of this material. This was presumed to be tetrahydrocannabinol. Compound No. 7 gave a spot which partly overlapped with that of No. 6. It was present in some, but not all, of the preparations of the phenolic fraction. Compounds Nos. 8 and 9 ( $R_f$  0.73 and 0.93) gave faint lemon-yellow spots and no visible absorption in the U. V.

**Partition Chromatography on Celite of the Phenolic Fraction.**—Celite 545 (diatomaceous earth) (150 Gm.) was thoroughly mixed with 75 ml. of the lower phase prepared by shaking together 1,500 ml. cyclohexane with 200 ml. of dimethylformamide. The Celite was packed in a column (37 × 580 mm.) the hold-back volume of which was 300 ml. The phenolic fraction, 1.448 Gm., was dissolved in the upper phase of the above solvent system and placed on the column. The upper (moving) phase of the solvent system was allowed to flow through the column at the rate of 2 ml. per minute. Cuts (25 ml.) were collected and washed twice with 75 ml. of water to remove the dimethylformamide. Three-tenths milliliter from each cut was diluted with 2.7 ml. of methanol and its absorption at 280  $m\mu$  was determined.

The two main peaks were well separated; the first, eluted in the second hold-back volume contained compound No. 6 with traces of No. 4. The second main peak, eluted in the third and fourth hold-back volumes, contained almost pure compound No. 4. Cuts were pooled as shown in Table II and the solvents removed *in vacuo*. The distribution of solids indicates that compound No. 6 (? tetrahydrocannabinol) was present in more than double the concentration of compound No. 4 (cannabinol) and constituted about 26% of the total solids in this preparation of the phenolic fraction.

In this preparation the amount of inert oil was high (cuts 1-5 total solids 494 mg.). To reduce the amount of inert material, 1.094 Gm. of phenolic fraction was dissolved in 25 ml. of the upper (cyclohexane) phase of the solvent system and extracted twice with 25 ml. of the lower (dimethylformamide)

<sup>2</sup> Assays of activity were carried out by Dr. G. Tonelli of the Experimental Therapeutics Research Section.

<sup>3</sup> The cannabidiol was kindly supplied by Dr. J. H. Clark and the cannabinol by Dr. Roger Adams.

TABLE II.—PARTITION OF PHENOLIC COMPONENTS OF *Cannabis sativa* ON CELITE BY MEANS OF CYCLOHEXANE/DIMETHYLFORMAMIDE<sup>a</sup>

Cuts	Solids, mg.	R <sub>f</sub> of DSA Spot
1-5	494	No color
6-7	23	No color
8-9	12	No color
11-19	382	0.56
23-32	182	0.37
35-38	Trace	0.16

<sup>a</sup> Cuts 25 ml., hold-back volume 300 ml., paper strips sprayed with diazotized sulfanilic acid (DSA).

phase. The lower phase contained 840 mg. of the solids which, after passage through a partition column as described above, yielded 360 mg. of compound No. 6 (43%). Other batches of the phenolic fraction prepared in this way gave similar yields of compound No. 6. The cannabiniol content of such fractions was approximately 10%. The amount of cannabidiol in these preparations was less than 1%.

Compound No. 6 (? tetrahydrocannabinol) as obtained in this way was active by the ataxia test in dogs. It was a colorless resin which rapidly acquired a purple tinge on exposure to air, later becoming yellow, and finally dark brown. Prevention of these color changes proved extremely difficult and even samples sealed under nitrogen became altered in this manner to some extent. The material also frequently contained traces of dimethylformamide, enough to give as much as 1% nitrogen on analysis.

**Sublimation, Analysis, and Physical Characteristics of Active Fraction.**—Further purification of the active fraction by sublimation at 97° at a pressure of  $<10^{-4}$  mm. Hg yielded a nitrogen-free product giving a single spot ( $R_f$  0.54) with the cyclohexane/dimethylformamide system. This colorless resin was solid at room temperature, had a specific rotation of  $[\alpha]_D^{25} - 161^\circ$ , U. V. absorption min. 251, max. 275, 282  $\mu$  ( $\log \epsilon$  3.26, 3.28) shifting in 0.1 *N* sodium hydroxide to min. 269, max. 292  $\mu$  ( $\log \epsilon$  3.53) with a second peak at 325  $\mu$ .

*Anal.*—Calcd. for  $C_{21}H_{30}O_2$  (tetrahydrocannabinol): C, 80.21; H, 9.62. Found: C, 79.13, 79.69; H, 9.89, 9.67.

The discrepancy between the found values for carbon and those calculated for tetrahydrocannabinol is rather large. Data for the U. V. absorption agree closely with that obtained by Wollner, *et al.*, for a preparation of tetrahydrocannabinol prepared from Indian charas via the acetate (peaks at 276  $\log \epsilon = 3.42$  and 280  $\log \epsilon = 3.43$ ).

Compound No. 4 crystallized on standing and proved, on comparison with a synthetic sample of cannabiniol from Dr. Roger Adams, to have an almost identical U. V. absorption spectrum. Natural cannabiniol max. 283, min. 248  $\mu$  ( $\log \epsilon$  4.22). Synthetic cannabiniol max. 283, min. 248  $\mu$  ( $\log \epsilon$  4.25). This compound in 0.1 *N* sodium hydroxide (methanolic) gave double maxima and minima.

Natural cannabiniol max. 283, min. 263  $\mu$  ( $\log \epsilon$  4.04, 3.28,  $\log \epsilon$  3.91, 3.10). Synthetic cannabiniol max. 284, min. 263  $\mu$  ( $\log \epsilon$  4.05, 3.28,  $\log \epsilon$  3.92, 3.10). The I. R. absorption spectra of natural tetrahydrocannabinol and cannabiniol are shown in Fig. 1.

The synthetic cannabiniol from Dr. Adams, which

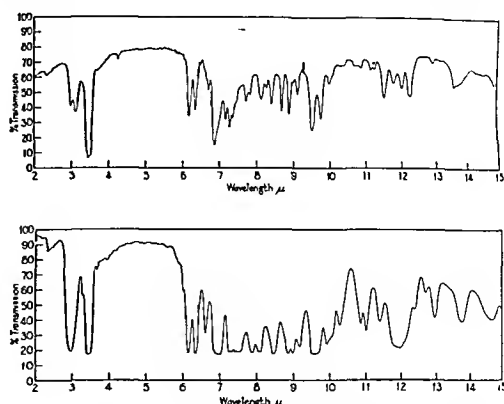


Fig. 1.—Infrared absorption spectra of natural cannabiniol (above) and tetrahydrocannabinol (below).

was the color and consistency of pitch when received after ten years' storage, was purified by passage through a cyclohexane/dimethylformamide column and yielded five impurities of various shades of crimson, orange, and purple. About 88% of the sample was still cannabiniol which indicates that this compound does not deteriorate as much as its change in color might suggest.

## SUMMARY

1. The solids from a methanolic extract of the flowering tops of *Cannabis sativa* were adsorbed on Florisil and eluted with benzene to yield a red oil producing ataxia in dogs at 10 to 25 mg./Kg.

2. Paper chromatography of the red oil using the system *N,N*-dimethylformamide and cyclohexane separated eight phenolic components giving a yellow or orange color with diazotized sulfanilic acid.

3. By partition chromatography on Celite using the above system the active fraction (tetrahydrocannabinol) was separated from the other components (cannabiniol, cannabidiol).

4. Further purification of the active fraction was achieved by high vacuum distillation.

5. The unstable colorless resin produced in this way could not be crystallized. Analyses were in fair agreement with the theoretical requirements for tetrahydrocannabinol ( $C_{21}H_{30}O_2$ ). The other components isolated from this sample of cannabis did not appear to have pharmacological activity.

## REFERENCES

- (1) Wollner, H. T., Matchett, J. R., Levine, J., and Loewe, S., *J. Am. Chem. Soc.*, **64**, 26(1942)
- (2) Adams, R., Baker, B. R., and Wearn, R. B., *ibid.*, **62**, 2204(1940).
- (3) Adams, R., Pease, D. C., and Clark, J. H., *ibid.*, **62**, 2194(1940).
- (4) Adams, R., Pease, D. C., Cain, L. K., and Clark, J. H., *ibid.*, **62**, 2402(1940).
- (5) Adams, R., Ayeock, B. F., and Loewe, S., *ibid.*, **70**, 662(1948).
- (6) Loewe, S., *Arch. Exptl. Pathol. Pharmacol. Naunyn-Schmiedeberg's*, **211**, 175(1950).

# Determination of Prednisolone in the Presence of Hydroxyzine and in Formulations Containing Analgesics\*

By J. D. DUERR and B. A. PAPPAS

A colorimetric method for the determination of prednisolone in the presence of hydroxyzine [1-(*p*-chlorobenzhydryl)-4-2-(2-hydroxyethoxy) ethyl piperazine], acetylsalicylic acid, caffeine, and acetophenetidin is proposed. Samples of tablet formulations containing prednisolone and the above mentioned compounds were assayed by the addition of sulfuric acid and ferric chloride to alcoholic solutions of the active ingredient and subsequent measurement of the absorbance of the resulting colored solutions.

THE MANUFACTURE of formulations containing prednisolone combined with tranquilizers or analgesics has been rapidly increasing. Prednisolone, when present alone in a product, is quite simple to determine. The addition of other compounds, however, increases the difficulty of quantitatively determining prednisolone using the existing assay procedures. Three methods in general use are: ultraviolet absorption (1); colorimetry, using triphenyltetrazolium chloride in an alkaline, alcoholic medium (2); and colorimetry, by the addition of concentrated sulfuric acid to dry prednisolone powder (3).

In the presence of hydroxyzine or the analgesics, simple ultraviolet absorbance measurements cannot be made without recourse to complicated extraction procedures. The color formed with triphenyltetrazolium chloride is destroyed by hydroxyzine or acetylsalicylic acid. Extracting the active ingredients, evaporating the solvent, and adding concentrated sulfuric acid to the residue gives erratic and inconsistent results. The proposed method eliminates complicated extractions and produces a stable color.

## EXPERIMENTAL

**Reagents.**—The following reagents were used throughout the assay procedure: (a) 3A alcohol (SDA 3A alcohol which is composed of 100 gallons of 190-proof ethyl alcohol + 5 gallons of methyl alcohol); (b) 10% w/v aqueous ferric chloride solution; (c) concentrated sulfuric acid (reagent grade); and (d) a solution of concentrated sulfuric acid + 3A alcohol (2:1).

**Absorption Maximum.**—A sample of standard prednisolone (100% purity by chromatography) was dissolved in 3A alcohol to give a concentration of 0.2 mg./ml. A 3-ml. aliquot containing 0.6 mg. of prednisolone was transferred to a 25-ml. glass-stoppered volumetric flask. Because of the heat evolved when the sulfuric acid was added to the

alcoholic solution of the active materials, the size and shape of the container was critical. It was necessary to use 25-ml. volumetric flasks to obtain reproducible results when following this assay procedure. The aliquot was diluted to 5 ml. with 3A alcohol. Five milliliters of 3A alcohol was transferred to a 25-ml. volumetric flask for the blank. Five milliliters of concentrated sulfuric acid was then added to the active aliquot and the blank while the flasks were being swirled to avoid spattering. The flasks were then stoppered and inverted but not shaken. They were then allowed to stand right-side-up at room temperature for exactly five minutes. Ferric chloride hexahydrate aqueous solution, 0.02 ml. of 10% w/v, was then added. The flasks were stoppered tightly and inverted a few times. They were then placed in a water bath at 20° for five minutes. At the end of this time, 5 ml. of 3A alcohol was added. The flasks were then shaken and allowed to stand in the 20° water bath for five minutes. The absorbance was read against the prepared reagent blank on a Cary recording spectrophotometer model 11. The absorption maximum was found to be at 525 mμ.

**Conformity to Beer's Law.**—Aliquots containing 0.2, 0.4, 0.6, 0.8 and 1.0 mg. of prednisolone were transferred to glass-stoppered 25-ml. volumetric flasks from the standard solution prepared for the determination of the absorption maximum. The aliquots were diluted to 5 ml. with 3A alcohol, and 5 ml. of 3A alcohol was used as the blank. The assay procedure used in determining the absorption maximum was followed. The absorbances of the active aliquots were read against the prepared reagent blank on a model B Beckman spectrophotometer. The absorbances obtained were plotted against their respective concentration per aliquot, and the straight line proved conformity to Beer's law. The assay results appearing in Tables I to III were calculated by running a reference standard at the time of assay.

**Assay of Laboratory Mixtures Containing Known Amounts of Prednisolone.**—Mixtures containing 30-60 mg. of prednisolone, 250 mg. hydroxyzine hydrochloride, 5 Gm. acetylsalicylic acid, 2.5 Gm. acetophenetidin, and 750 mg. of caffeine were transferred to 250-ml. volumetric flasks. 3A Alcohol was added about three-fourths to mark and the mixtures shaken for fifteen minutes. The flasks were then diluted to volume with 3A alcohol.

\* Received January 13, 1960, from Chas. Pfizer & Co., Inc., Process Development Laboratory, Pharmaceutical Production and Packaging Dept., Brooklyn, N. Y.

The solutions were filtered through Whatman No. 2 filter paper and aliquots containing 0.3, 0.4, and 0.5 mg. were transferred to 25-ml. glass-stoppered volumetric flasks. The assay procedure used in the determination of the absorption maximum was followed. The recovery figures of prednisolone from the synthetic mixtures were calculated from a reference solution of prednisolone standard in 3A alcohol and appear in Table I.

TABLE I.—RECOVERY OF PREDNISOLONE FROM LABORATORY MIXTURES CONTAINING KNOWN AMOUNTS OF PREDNISOLONE, ACETYSALICYLIC ACID, ACETOPHENETIDIN, AND CAFFEINE

Laboratory Blend No.	Prednisolone Theoretical Content, Gm.	Assay, Gm.	Recovery, %
1	0.0403	0.0398	98.7
2	0.0504	0.0500	99.2
3	0.0302	0.0303	100.3
4	0.0500	0.0501	100.2
5	0.0600	0.0601	100.1
6	0.0251	0.0254	101.1
7	0.0354	0.0354	100.0

**Assay of Prednisolone Tablets.**—A known quantity of tablets were weighed to determine the average weight of one tablet. They were then ground to a fine powder. A sample of the powder was shaken with 3A alcohol for fifteen minutes and then diluted to contain 0.2 mg./ml. of prednisolone. Aliquots containing approximately 0.2, 0.4, 0.6, and 0.8 mg. of prednisolone were transferred to glass-stoppered 25-ml. volumetric flasks. The aliquots were diluted to 5 ml. with 3A alcohol, and 5 ml. of 3A alcohol was used for the blank. The assay procedure used in determining the absorption maximum was then followed. The results of the assays are shown in Table II.

TABLE II.—ASSAY OF PREDNISOLONE TABLETS

Sample No.	Label, mg./Tablet	Assay, mg./Tablet
1	0.50	0.491
2	0.50	0.501
3	0.50	0.502
4	0.50	0.504
5	0.50	0.499
6	0.50	0.498
7	0.50	0.502

**Assay of Prednisolone in the Presence of Hydroxyzine.**—A quantity of tablets containing prednisolone and the tranquillizer hydroxyzine were weighed to determine the average weight of one tablet. They were then ground to a fine powder. A sample of the powder containing approximately 50 mg. of prednisolone was transferred to a 250-ml. volumetric flask. 3A alcohol was added half way to mark and the mixture was shaken for fifteen to twenty minutes. The flask was then brought to mark with 3A alcohol. After filtering through paper, the same procedure was followed as in the assay for prednisolone tablets. The results of the assays are shown in Table III.

TABLE III.—DETERMINATION OF PREDNISOLONE IN TABLETS CONTAINING THE APC FORMULA OR HYDROXYZINE

	Lot No.	Label, mg./Tablet	Assay, mg./Tablet
Prednisolone + Hydroxyzine	1	1.00	1.00
	2	1.00	1.00
	3	1.00	1.10
	4	2.50	2.70
	5	2.50	2.60
	6	5.00	5.10
	7	5.00	5.08
	8	5.00	5.10
	9	5.00	4.98
	10	5.00	4.90
	11	5.00	5.00
	12	5.00	5.00
	13	5.00	5.10
Prednisolone + APC	1	0.50	0.49
	2	0.50	0.51
	3	0.50	0.50
	4	0.50	0.50
	5	0.50	0.51
	6	0.50	0.51

**Assay of Prednisolone in Prednisolone APC Tablets.**—A quantity of tablets containing prednisolone, aspirin, acetophenetidin, and caffeine were weighed to determine the average weight of one tablet. The same procedure was then followed as in the assay of prednisolone tablets. The results of the assays appear in Table III.

#### ALTERNATE METHOD

Although the procedures discussed give highly satisfactory results, an alternate method was devised in the event the assayist preferred to dilute the reaction mixture to a known volume. Aliquots containing 400 to 1,000 mcg. were transferred to 25-ml. volumetric flasks or graduated glass-stoppered tubes. The aliquots were diluted to 5 ml. with SDA 3A alcohol. Five milliliters of SDA 3A alcohol was used for a blank. Five milliliters of concentrated sulfuric acid was then added to the active aliquots and the blank while the containers were being swirled to avoid spattering. The containers were then stoppered and inverted but not shaken. They were then allowed to stand for exactly five minutes. Ferric chloride hexahydrate aqueous solution, 0.2 ml. of 10% w/v, was then added. The containers were stoppered tightly and inverted a few times. They were then placed in a water bath at 20° for five minutes. At the end of this time the solutions were diluted to 25 ml. with a mixture containing 10 ml. of concentrated sulfuric acid plus 5 ml. of SDA 3A alcohol. The containers were then shaken and allowed to stand in a water bath at 20° for five minutes. The absorbance was then read against the prepared reagent blank on a Cary recording spectrophotometer model 11. The absorption maximum was found to be at 525 mμ. The assay results appearing in Tables IV-VI were calculated by running a reference standard at the time of assay.

**Assay of Laboratory Mixtures Containing Known Amounts of Prednisolone.**—Mixtures containing 30–50 mg. of prednisolone, hydroxyzine hydrochloride, acetylsalicylic acid, acetophenetidin, and cal-

TABLE IV.—RECOVERY OF PREDNISOLONE FROM LABORATORY MIXTURES CONTAINING HYDROXYZINE, CAFFEINE, ACETYSALICYLIC ACID, AND ACETOPHENETIDIN USING ALTERNATE METHOD

Laboratory Blend No.	Prednisolone Theoretical Content, mg.	Assay, mg.	Recovery, %
1	25.2	26.0	103.2
2	50.0	51.6	103.0
3	50.3	50.0	99.0
4	46.0	45.8	99.6
5	35.0	35.1	100.3
6	61.0	61.2	100.3

TABLE V.—ASSAY OF PREDNISOLONE TABLETS

Sample No.	Label, mg./Tablet	Assay, mg./Tablet
1	5.0	4.9
2	5.0	5.1
3	5.0	4.9

TABLE VI.—DETERMINATION OF PREDNISOLONE IN TABLETS CONTAINING THE APC FORMULA OR HYDROXYZINE

	Sample No.	Label, mg./Tablet	Assay, mg./Tablet
Prednisolone + hydroxyzine	1	1.0	1.1
	2	2.5	2.5
	3	2.5	2.6
	4	5.0	5.2
	5	5.0	5.2
Prednisolone + APC	1	0.5	0.53
	2	0.5	0.49
	3	0.5	0.50

feine were transferred to 250-ml. volumetric flasks and dissolved with SDA 3A alcohol. The flasks were then diluted to mark with SDA 3A alcohol. The solutions were then assayed using the alternate method; the recovery figures of prednisolone appear in Table IV.

**Assay of Tablets Containing Prednisolone.**—The tablets were prepared as in the first method and then assayed as outlined in the alternate method. The results appear in Table V.

**Assay of Prednisolone in the Presence of Hydroxyzine.**—The tablets were prepared as in the first method and assayed as outlined in the alternate method. The results appear in Table VI.

**Assay of Prednisolone in Presence of the APC Formula.**—The tablets were prepared as in the first method and then assayed as outlined in the alternate method. The results appear in Table VI.

## DISCUSSION

Since it seemed obvious that steroids other than prednisolone ( $\Delta 1,4$ -pregnadiene-11 $\beta$ , 17 $\alpha$ , 21-triol-3,20-dione) would interfere with this assay, the color and absorption maxima of several commonly-used steroids were obtained using the procedure described above. Estrone (1,3,5-estratriene-3-ol-17-one) gave a pinkish-red color with an absorption maximum at 523  $m\mu$ , estradiol (1,3,5-estratriene-3, 17 $\beta$ -diol) a pink-red color with a maximum at 533  $m\mu$ , testosterone ( $\Delta 4$ -androstene-17-ol-3-one) a green color with a maximum at 468  $m\mu$ . It is interesting to note that prednisone ( $\Delta 1,4$ -pregnadiene-17 $\alpha$ ,21-diol-3,11,20-trione), which is different from prednisolone only in having a keto group at position 11, does not produce a color and shows no definite absorption maximum between 400 and 700  $m\mu$  when tested at concentrations up to 0.5 mg. per aliquot.

## SUMMARY

1. Prednisolone concentration can be accurately determined by forming a chromagen in alcoholic solution with concentrated sulfuric acid and aqueous ferric chloride with subsequent measurement of the absorbance on a suitable spectrophotometer.

2. The methods are also applicable to formulations containing hydroxyzine, acetylsalicylic acid, acetophenetidin, and caffeine.

3. The substances mentioned above which do interfere with other assay procedures do not have to be separated from the prednisolone prior to assaying.

## REFERENCES

- (1) Herzog, H. L., Nobile, A., Tolksdorf, S., Cbarney, W., Hersbberg, E. B., and Perlman, P. L., *Science*, 121, 176 (1955).
- (2) Mader, W. J., and Buck, R. R., *Anal. Chem.*, 24, 666 (1952).
- (3) Conca, N., private communication, Chas. Pfizer & Co., Brooklyn, N. Y., 1956



# Metabolism of Dextrosulphenidol in Several Animal Species\*

By EVAN W. MCCHESENEY, RAYMOND F. KOSS, JAMES M. SHEKOSKY, and WILLIAM H. DEITZ

The *in vivo* metabolism of dextrosulphenidol has been studied using various test organisms. Differences are noted and comparisons are made as to the effect of biological variation on the absorption, distribution, and excretion of the drug.

INVESTIGATION of the metabolism of dextrosulphenidol<sup>1</sup> (DES) has thus far been limited by the lack of adequate chemical assay methods, the data which are presently available having been obtained by microbiological assay (tube dilution techniques). Using these methods, Shaffer, *et al.* (1), have shown DES to be well absorbed by chicks when administered orally; nevertheless, concentrations which were inhibitory to the test organism remained in the intestinal tract for several hours after a dose of 100 mg./Kg. With doses of 400 mg./Kg., serum levels of 31–62 mcg./ml. were maintained for as long as three to six hours, and levels of 4–8 mcg./ml. were detected as late as twelve hours postmedication. Drug levels in the bile were higher and more prolonged than those in the plasma. Using similar methods, one of us (W. H. D.) has studied the absorption of DES in dogs and man (2). Single doses of 75 and 150 mg./Kg. have been given to mongrel dogs; the former dose produced peak serum levels of 50 mcg./ml. at two and four hours, and by twelve hours postmedication this level had fallen to 3 mcg./ml. The latter dose gave a four-hour level of 100 mcg./ml. and a twelve-hour level of 7 mcg./ml. Dogs also received and tolerated well doses of 20, 40, or 80 mg./Kg. twice daily for twenty-one days. The initial doses resulted in two-hour serum levels of 6, 10, and 22 mcg./ml., respectively. In man, the administration of a single dose of 1 Gm. of DES resulted in a serum level of 6 mcg./ml. at one and one-half hours. It was observed in three subjects that man excretes about 70 per cent of an oral dose of 500 mg., in biologically active form, within twenty-two hours.

The development of a chemical procedure for the determination of DES (3) has made possible

a more detailed study of its metabolism. This procedure consists essentially of ethyl acetate extraction, alkaline hydrolysis, oxidation with periodate at pH 7.5, and final estimation of *p*-methylsulfonylbenzaldehyde as an alkali salt of its *p*-nitrophenylhydrazone. Data on the absorption, tissue distribution, and excretion of DES in rat, dog, cat, rabbit, and man are presented in this paper.

## EXPERIMENTAL

Albino rats weighing 170–230 Gm. were fasted for at least eight (but not more than fifteen) hours prior to medication and during the entire metabolic period, but they had access to water at all times. After medication they were placed in metabolism cages, for the quantitative collection of the excreta. Generally speaking, no feces were eliminated in the brief period studied, and the urine volume was rather low. At various intervals postmedication the animals were sacrificed by decapitation, and representative tissues were removed for analysis. These included heart, lung, liver, kidney, spleen, and muscle; blood plasma was also obtained at this time. The urine was analyzed for both free and total DES as previously described (3); the stomach plus content, and the intestines plus content were analyzed separately.

Both oral and intravenous administration were studied in the rat. To improve solubility, the compound was dissolved in 50% propylene glycol; the dose of DES was 50 mg./Kg. and the dose of glycol was 2,500 mg./Kg. The results of these experiments are presented in Table I.

Similar data on the metabolism of DES in other animal species were obtained in one dog, one cat, and two rabbits. In order to obtain strictly comparable data on the several species, all of the animals were fasted for eight hours prior to medication, and during the sixteen-hour metabolic period but, again, they had access to water at all times. The oral medications (50 mg./Kg.) were given in 50% propylene glycol, the total dose of the glycol again being 2,500 mg./Kg. Since these are the same experimental conditions as were used for one of the groups of orally medicated rats, the data on the individual animals of this specific group are also given for comparative purposes. These data are presented in Table II.

One laboratory volunteer took a single 500-mg./dose (two tablets) of DES and collected urine under toluene for six periods comprising the next forty-eight hours. These samples were analyzed both chemically and microbiologically (using *P. septica* as the test organism), with the results presented in Table III.

\* Received March 1, 1960, from the Sterling-Winthrop Research Institute, Rensselaer, N. Y.

<sup>1</sup> In the earlier literature, dextrosulphenidol, or *nd*-threo-2-dichloroacetamido-1-(4-methylsulfonylphenyl)-1,3-propanediol, was referred to as Thioeymetin. This trade name is now reserved for the *dl*-form (racephenidol).

TABLE I—ABSORPTION, TISSUE DISTRIBUTION, AND EXCRETION OF DEXTROSULPHENIDOL IN RATS RECEIVING DOSES OF 50 MG/KG<sup>a</sup>

Material Analyzed	Hours after Medication						T/P Ratio <sup>c</sup>
	2	4	8	16	16 <sup>b</sup>	24	
	Values recorded as mcg/Gm (mean $\pm$ S.E.)						
Heart	7.4 $\pm$ 0.4	4.0 $\pm$ 0.5	1.5 $\pm$ 0.8	0.5 $\pm$ 0.5	1.9 $\pm$ 0.9	1.4 $\pm$ 0.8	2.7 $\pm$ 0.8
Lung	7.9 $\pm$ 0.5	3.9 $\pm$ 0.8	1.0 $\pm$ 0.4	1.6 $\pm$ 0.5	0.9 $\pm$ 0.3	0.7 $\pm$ 0.2	2.1 $\pm$ 0.4
Spleen	21 $\pm$ 7.4	41 $\pm$ 23	26 $\pm$ 19	19 $\pm$ 16	13.1 $\pm$ 1.3	1.5 $\pm$ 2.0	11.2 $\pm$ 6.9
Kidney	29 $\pm$ 2.3	48 $\pm$ 30	8.2 $\pm$ 2.3	12 $\pm$ 2.9	1.7 $\pm$ 0.5	3.3 $\pm$ 0.8	12.8 $\pm$ 2.8
Liver	9.7 $\pm$ 1.2	12 $\pm$ 3.8	2.5 $\pm$ 1.4	4.9 $\pm$ 1.1	1.3 $\pm$ 0.5	1.5 $\pm$ 0.4	4.9 $\pm$ 0.9
Muscle	6.8 $\pm$ 2.1	4.7 $\pm$ 0.7	2.6 $\pm$ 1.1	0.9 $\pm$ 0.2	0.3 $\pm$ 0.1	0.3 $\pm$ 0.3	2.0 $\pm$ 0.5
Plasma	7.5 $\pm$ 0.3	2.8 $\pm$ 0.7	0.6 $\pm$ 0.02	0.6 $\pm$ 0.01	0.1 $\pm$ 0.1	0.5 $\pm$ 0.1	
Total Dextrosulphenidol Present, mcg (Mean $\pm$ S.E.)							
Heart	5.0 $\pm$ 0.6	3.0 $\pm$ 0.4	1.0 $\pm$ 0.6	0.3 $\pm$ 0.3	1.7 $\pm$ 0.8	1.2 $\pm$ 0.7	
Lung	12 $\pm$ 2.1	5.0 $\pm$ 1.1	2.0 $\pm$ 0.4	2.0 $\pm$ 0.6	1.2 $\pm$ 0.4	1.0 $\pm$ 0.3	
Spleen	15 $\pm$ 5.0	37 $\pm$ 26	18 $\pm$ 10	12 $\pm$ 9	2.8 $\pm$ 1.2	0.8 $\pm$ 0.8	
Kidney	60 $\pm$ 10	83 $\pm$ 55	14 $\pm$ 5	18 $\pm$ 4	3.0 $\pm$ 1.0	5.0 $\pm$ 1.5	
Liver	68 $\pm$ 12	69 $\pm$ 30	28 $\pm$ 11	30 $\pm$ 6	8.5 $\pm$ 3.0	8.2 $\pm$ 2.5	
Muscle	522 $\pm$ 108	380 $\pm$ 46	195 $\pm$ 96	67 $\pm$ 20	34 $\pm$ 14	24 $\pm$ 24	
Plasma	30 $\pm$ 1.2	11 $\pm$ 3	2.0 $\pm$ 0.1	1.9 $\pm$ 0	0.3 $\pm$ 0.3	1.7 $\pm$ 0.1	
Stomach	160 $\pm$ 50	30 $\pm$ 10	10 $\pm$ 5	5.0 $\pm$ 2	4.0 $\pm$ 1.2	2.0 $\pm$ 0.6	
Intestine	4,000 $\pm$ 400	3,320 $\pm$ 120	2,350 $\pm$ 310	1,400 $\pm$ 230	460 $\pm$ 118	750 $\pm$ 15	
Urine							
Free	1,070 $\pm$ 410	1,765 $\pm$ 185	2,410 $\pm$ 47	4,247 $\pm$ 85	6,960 $\pm$ 1,270	4,870 $\pm$ 236	
Total	1,070 $\pm$ 395	1,830 $\pm$ 56	2,510 $\pm$ 109	4,298 $\pm$ 115	6,910 $\pm$ 1,100	4,730 $\pm$ 250	
Feces	0	0	0	0	0	0	
Per Cent of Dose Present							
Tissues <sup>d</sup>	8.4 $\pm$ 1.7	6.7 $\pm$ 1.9	3.2 $\pm$ 1.5	1.5 $\pm$ 0.5	0.5 $\pm$ 0.2	0.5 $\pm$ 0.4	
G.I. tract <sup>e</sup>	49.2 $\pm$ 5.3	39.4 $\pm$ 1.5	28.9 $\pm$ 3.8	16.5 $\pm$ 2.7	4.2 $\pm$ 1.1	8.8 $\pm$ 0.2	
Urine	12.7 $\pm$ 4.7	21.5 $\pm$ 0.7	30.8 $\pm$ 1.3	53.8 $\pm$ 1.4	63.0 $\pm$ 10.0	56.8 $\pm$ 3.0	

<sup>a</sup> The medication was oral except where otherwise indicated. <sup>b</sup> Intravenous medication. <sup>c</sup> Ratio of tissue concentration to plasma concentration determined concomitantly, mean  $\pm$  S.E. for 16 animals. Two sixteen hour i.v. animals with no detectable plasma level are excluded. <sup>d</sup> Total in the tissues analyzed. <sup>e</sup> In stomach, intestine, cecum.

TABLE II—COMPARATIVE METABOLISM OF DEXTROSULPHENIDOL IN SEVERAL ANIMAL SPECIES<sup>a b</sup>

Material Analyzed	Species						
	Rat	Rat	Rat	Dog	Cat	Rabbit	Rabbit
Heart	0	0	1 5	2 1	4 9	0 2	1 4
Lung	0 8	1 6	2 4	0 4	5 8	0 2	0 3
Spleen	5 9	1 5	50 0	0 8	4 8	0 6	2 0
Kidney	14 5	5 9	14 8	2 7	9 4	4 0	2 0
Liver	2 9	5 1	6 8	0 8	9 7	0 4	2 4
Muscle	0 4	1 1	1 1	1 3	3 1	0 3	0 7
Brain				0 7	2 9	1 2	1 2
Pancreas				1 0			
Fat				0		1 0	0 6
Bile				30	16	74	52
Plasma	0 6	0 7	0 6	0 7	4 9	0 5	0 5
Dextrosulphenidol Present, mg							
Stomach	0 003	0 06	0 08	0 03	0 06	0 54	0 41
Small intestine				0 12		0 93	0 48
Cecum-colon	1 57	1 68	0 94	2 78	16 0	6 45	7 50
Urine							
Free DES	4 30	4 08	4 70	200 0	52 5	43 5	44 4
Total DES	4 58	3 96	4 60	235 0	52 3	43 8	42 5
Feces	0	0	0	10 8	0	0	0
Dose, mg	7 9	8 5	7 6	350 0	107 0	137 5	133 0
Dose in urine, %	54 8	47 1	61 2	67 3	49 0	31 9	31 9

<sup>a</sup> All observations made sixteen hours after a single oral dose of 50 mg/Kg<sup>b</sup> Values recorded as mcg/Gm.<sup>c</sup> Indicates material not analyzed

TABLE III—EXCRETION AND METABOLISM OF DEXTROSULPHENIDOL IN MAN FOLLOWING A SINGLE ORAL 500-MG DOSE

Interval of Urine Collection, hr	Volume Output, ml	Chemical Assay				Microbiological Assay <sup>c</sup>	
		Free DES, mcg/ml	Total DES, <sup>a</sup> mcg/ml	Mg Excreted in interval <sup>b</sup>	Mg Excreted per hr	Mcg/ml	Mg Excreted in Interval
0-4	126	742	732	92 5	23	800	101
4-8	106	1,130	1,130	120 0	30	1200	127
8-12	156	410	420	64 7	16	400	62
12-24	343	184	175	61 7	5	200	69
24-32	230	101	110	25 2	3	100	23
32-48	740	11	13	8 9	0 6	20	15
Totals	1,701			373			397
Means		222	221		7 8	234	

<sup>a</sup> Analysis involved preliminary drastic alkaline hydrolysis<sup>b</sup> Based on mean of free and total DES values<sup>c</sup> Determined by serial dilution techniques, using *P. septicus* as the test organism

## DISCUSSION

DES demonstrates in all of the animal species studied the typical metabolic pattern of a compound which is well absorbed, circulates freely in the extracellular fluids, and is excreted in both urine and bile. It has no outstanding tissue predilection except possibly for the spleen, and this exception has been observed in only one of the species (the rat). General comments on the results on the individual species studied follow.

**The Rat**—At the early intervals, the concentrations of DES in heart, lung, and muscle are essentially the same as those existing concurrently in the plasma. The concentrations in the kidney and liver are definitely higher than those existing concomitantly in the plasma, as would be expected for the organs which are directly involved in the excretory processes. The tissue/plasma concentration ratios in the spleen are very erratic, varying from 2 to 80, even in two animals from the same group (the sixteen-hour, oral). A relatively high

splenic concentration is also characteristic of chloramphenicol metabolism (4). The biological half-life of DES in the rat, as estimated from the decreases in tissue and plasma levels with time, is about five hours; the similarly estimated value for chloramphenicol is one and one-half to three hours (5).

It is evident from the data that the rat excretes DES in the urine entirely in unchanged form, since in no group of animals is there a significant difference between free and total DES. This eliminates the possibility that the rat excretes, via the urine, any measurable amount of DES as a glucuronide or as the free base.<sup>2</sup> Both of these derivatives have been detected in the urine following the administration of chloramphenicol (6). The urinary excretion of DES increases with time, to a maximum of 57% of the dose, in twenty-four hours. It is of interest to note, however, that the sums of the amounts present in the urine and the digestive tract remain nearly

<sup>2</sup> That is, and three 1-(4-methylsulfonylphenyl) 1,3-propanediol

constant during the twenty-four-hour period, all of these sums being between 59 and 70% of the dose. While the animal is effecting this transfer of DES from intestine to urine, the amounts in the tissues decrease steadily, from 8.5% of the dose at two hours, to 0.5% at twenty-four hours. A considerable part of the dose (about 30%) is not accounted for and must be present in the tissues or the digestive tract in a form which is not extractable and, therefore, not determinable by the analytical method used. Extensive metabolic degradation is clearly ruled out as a possibility.

The results on the intravenously medicated animals demonstrate that excretion into the intestine definitely occurs. Residues in the intestine of the orally medicated animals, therefore, do not represent, exclusively, unabsorbed material. Tissue levels in the i. v. animals, sixteen hours after medication, are as low as those observed in the twenty-four-hour orally medicated animals, indicating a more rapid physiological disposition of the compound when it is administered parenterally. The higher level of urinary excretion in the intravenously medicated animals, as compared to the orally medicated animals sacrificed at the same time interval, points to the same conclusions.

Some possibilities for the fate of the unaccounted-for remainder (about 30%) of the orally or intravenously administered DES are as follows: (a) It may be excreted in the urine, but at a later time. This seems improbable in view of the fact that only 0.5% of the dose is found in the tissues analyzed at twenty-four hours postmedication; nevertheless, the point was studied by administering 50 mg./Kg. of DES orally in 50% propylene glycol to three pairs of rats (200–250 Gm. each) and collecting excreta for forty-eight hours thereafter. These rats had been fasted for fifteen hours prior to medication, but food was provided during the experimental period. The results of this experiment are presented in Table IV, and they demonstrate that delayed urinary excretion does not account for a very large proportion of the dose. However, it is also clear that the results in Table IV are not entirely comparable to those presented in Table I. The presence of food in the intestine at any time evidently alters the absorption and excretion of DES very materially, probably by stimulating the flow of bile. The result is that much more of the drug is excreted in the feces and much less in the urine of the fed animals than is the case when the animals receive no food whatsoever, after eight hours premedication.

TABLE IV.—URINARY AND FECAL EXCRETION OF DEXTROSULPHENIDOL IN THE RAT FOLLOWING AN ORAL DOSE OF 50 MG./KG.<sup>a</sup>

Group <sup>b</sup>	In Urine, Total DES		In Feces 0–48 hr.		Total Re- covered
	0–24 hr.	24–48 hr.	Free DES	Total DES	
A	38.7	5.7	37.8	54.4	98.8
B	31.9	4.6	36.6	49.5	86.0
C	41.1	4.6	29.8	40.0	85.7
Mean	37.2	5.0	34.7	48.0	90.2

<sup>a</sup> All values in the table are given as % of dose. <sup>b</sup> Two rats per group.

(b) It may be excreted in the bile as a glucuronide, a form which would not be extensively recycled and which would not be determined under the conditions used for DES analysis (3). This type of excretion has been demonstrated for chloramphenicol (7), but there is no entirely unambiguous procedure by which its occurrence can be demonstrated for DES, since no sample of its glucuronide is available for chemical or biological study. The point has been studied in the following way, however, based on the assumed properties of such a glucuronide:

Finely powdered rat feces (obtained from the animals of Table IV) were suspended in 100 parts of methanol, the suspension was adjusted to pH 3.5 with hydrochloric acid, digested on the steam bath for fifteen minutes, and filtered. Two aliquot portions of this extract were evaporated separately to dryness and were analyzed for free and total DES in a manner analogous to that used for urine (3): one of the residues was partitioned between ethyl acetate and phosphate buffer of pH 6.2, and the amount of DES in the ethyl acetate was determined; the other residue was subjected to a direct alkaline hydrolysis, followed by a determination of the free DES base<sup>3</sup> in the hydrolysate.

In all of the extracts so studied the "total" DES exceeded the "free" (i. e., chemically unaltered) by 25–35%. When the values for total fecal DES are calculated on this basis (Table IV, column 4) it is found that the total recovery becomes 85–99% of the amount administered. Since the procedure used to extract the supposed DES glucuronide from the feces is incapable of direct experimental verification, it cannot be assumed that such a procedure would necessarily extract the supposed glucuronide quantitatively and it is not necessary, therefore, to assume the existence of still another metabolic product to account for the remainder of the dose (in groups B and C Table IV).

(c) It may be converted to the free base by loss of the dichloroacetyl group (6) and the base may be further degraded by oxidative processes. This point was studied by administering the base<sup>3</sup> intravenously (35 mg./Kg.) to two groups of three rats each. Excreta were collected as described above and the base was determined in the urine as for total DES (3), except that the alkaline hydrolysis was omitted. The finely ground feces were extracted with methanol, followed by alkaline hydrolysis of the residue so extracted. The results are presented in Table V: they demonstrate that no substantial oxidation of the propanediol side chain occurs, and that the excretion of the base is largely renal.

The results described in sections (a) and (c) of this discussion may be interpreted to mean that either DES base or DES glucuronide is excreted in the bile and feces of rats. Of these possibilities, the latter is much more likely, in view of the data of Table V. The data of Table I fit readily into a logical scheme if it is assumed that about 65% of the administered DES is eventually excreted in the urine of (fasting) rats, and 35% is excreted in the bile as a glucuronide. The latter would not be

<sup>3</sup> Kindly supplied by Mr. S. Schalit of this Institute. The product used was the crystalline *dl*-form, m. p. 123–127°, % N = 5.82.

TABLE V.—URINARY AND FECAL EXCRETION<sup>a</sup> OF DEXTROSULPHENIDOL BASE<sup>b</sup> IN THE RAT FOLLOWING AN INTRAVENOUS DOSE OF 35 MG./KG.

Group <sup>c</sup>	In Urine		In Feces, 0-48 hr.	Total Re- covered
	0-24 hr.	24-48 hr.		
A	80.0	7.5	5.7	93.2
B	72.0	6.7	5.8	84.5

<sup>a</sup> All values in the table are as % of dose. <sup>b</sup> See text footnote 3. <sup>c</sup> Three rats per group

determinable in feces or intestinal content, except as described in (b).

*The Cat.*—So far as it is justifiable to draw conclusions from one animal, it may be said that the metabolism of DES in the cat differs significantly from that in the dog, rat, and rabbit. For example, the tissue levels in the cat sixteen hours postmedication are higher than those in any of the other species studied. However, the plasma level is correspondingly high, with the result that the tissue/plasma ratios remain nearly 1/1, except for the two organs directly involved in the excretory processes. It is of interest to note that the ratio of biliary to plasma concentration in the cat is only 3/1, as compared to 100/1 for the rabbit and 40/1 for the dog. The relatively low level of biliary excretion in the cat may correlate with the high tissue levels in this species, since its sixteen-hour urinary excretion is at least average for the animals studied.

*The Dog.*—Only in the dog is there a definite difference between free and total urinary DES. The sixteen-hour renal excretion is the highest of any of the species studied, and the amount remaining in the intestine of the dog is correspondingly low. The tissue levels in the dog are comparable to those of the twenty-four-hour rats (Table I, column 6) and of the sixteen-hour rabbits. The only DES found in the feces of the dog, however, is in an (analytically) unchanged form.

*The Rabbit.*—The results on this species differ most noticeably in the presence of considerable drug residues in the stomach, and in the low urinary excretion. These appear to be interrelated observations since, in spite of the fact that the rabbits had been fasted for eight hours prior to medication, very bulky food residues remained in all levels of their digestive tracts. It has been noted above that the presence of such residues affects the absorption and excretion rates of DES appreciably. Biliary excretion is evidently a very important process in the rabbit.

*Man.*—The only determinable form of DES excreted in the urine of man is the unchanged drug, as is shown by the fact that the chemically-determined values for free and total drug exactly coincide (see Table III). These observations are further confirmed by the fact that the microbiological assay of each sample is in very close agreement with the chemical analysis for total DES. No significant amount of glucuronide (determinable by difference), therefore, can be present, although this is the principal pathway of chloramphenicol metabolism

(6). The total urinary excretion of DES in forty-eight hours (in this one subject) is 75% of the dose, which contrasts markedly with the 5-6% of chloramphenicol so excreted (8-11).<sup>4</sup> Such excretion of (unchanged) chloramphenicol as does occur, furthermore, is mostly in the first six hours after administration.

## SUMMARY

The metabolism of dextrosulphenidol has been studied in the rat, dog, cat, rabbit, and man. In all species but one (the dog) the only urinary excretory product is the unchanged drug; in this one species about 15 per cent of the dose is evidently excreted in a conjugated form. Absorption and excretion of dextrosulphenidol appear to be delayed by the presence of food residues in the digestive tract; in their absence the urinary excretion in sixteen hours, regardless of the species, is about 60 per cent of the dose. Biliary excretion is also an important process, with the result that the drug continues to be found in the intestine as long as it is present in the tissues. Tissue:plasma concentration ratios are generally about 1:1 except in the liver and kidney, and possibly the spleen. There is some evidence that the drug is present in the intestine of the rat other than as unchanged dextrosulphenidol. This form could be either the free base or a glucuronide conjugate, but is more likely the latter. In man 75 per cent of an oral dose is excreted in unchanged form within forty-eight hours.

## REFERENCES

- (1) Shaffer, M. F., Milner, K. C., Clemmer, D. I., Potash, L., and Shaffer, L. S., *Antibiotics & Chemotherapy*, 4, 992 (1954).
- (2) Deitz, W. H., unpublished data, Sterling-Winthrop Research Institute.
- (3) McChesney, E. W., Shekosky, J. M., Eckert, H. W., and Koss, R. F., *THIS JOURNAL*, 49, 28 (1960).
- (4) Levine, J., and Fishbach, H., *Antibiotics & Chemotherapy*, 1, 59 (1951).
- (5) Orr, W. W., Preisser, W. G., Ross, S., Burke, F. G., and Rice, E. C., *ibid.*, 1, 63 (1951).
- (6) Glazko, A. J., Dill, W. A., and Rebstock, M. C., *J. Biol. Chem.*, 183, 679 (1950).
- (7) Glazko, A. J., Dill, W. A., and Wolf, L. M., *J. Pharmacol. Exptl. Therap.*, 104, 452 (1952).
- (8) Glazko, A. J., Wolf, L. M., Dill, W. A., and Bratton, A. C., *ibid.*, 96, 445 (1949).
- (9) Ley, H. L., Jr., Smadel, J. E., and Crocker, T. T., *Proc. Soc. Exptl. Biol. Med.*, 68, 9 (1948).
- (10) Shick, O., Cholinsky, K., Smadel, O., and Graff-Netterova, J., *Antibiotic Med. & Clin. Therapy*, 6, 98 (1959).
- (11) Trivellato, E., and Vettori, G., *Minerva med.*, 48, 4115 (1957).
- (12) Kunin, C. M., and Finland, M., *Proc. Soc. Exptl. Biol. Med.*, 103, 246 (1960).

<sup>4</sup> Recently Kunin and Finland (12) have studied comparatively the absorption and excretion of chloramphenicol and dextrosulphenidol in man. They found that an oral dose of 500 mg. of the latter gave a peak blood level of 4-6 mcg./ml. at two hours postmedication, and a cumulative forty-eight-hour urinary excretion (in six subjects) of  $336 \pm 42$  mg., when assayed against *S. lutea*.

# Prediction of Stability in Pharmaceutical Preparations VII\*

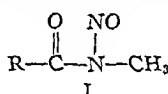
## The Solution Degradation of the Antibiotic Streptozotocin

By EDWARD R. GARRETT

The kinetics of solution degradation of the antibiotic streptozotocin have been studied polarographically, spectrophotometrically, and by bioassay. Equations have been obtained to characterize degradation as a function of pH, buffer, and temperature. Bioassay has been correlated with these others and correction procedures devised to account for their limitations. Stability *in vitro* and *in vivo* has been predicted. A mechanism is proposed that accounts for the available data and describes the weakly basic nature of streptozotocin and indicates the presence of a N-nitrosomethylurea function in the antibiotic.

THE PHARMACEUTICAL need to determine the stability of molecules of unknown structure has been discussed previously (1).

Streptozotocin is a new broad-spectrum antibiotic (2) of presently unknown structure but of suggested empirical formula,  $C_{14}H_{27}N_5O_{12}$ , with an indicated N-nitrosomethylamide group (I) (3).



Some chemical, physical (3), and biological (2, 4-6) properties of streptozotocin have been recently described.

This paper reports on studies designed to determine the stability of streptozotocin as a function of pH and temperature, to predict the nature of the intermediates or products of degradation, to compare chemical and biological assay procedures, and to predict stability *in vivo* and the conditions for maximum stability in solution.

### EXPERIMENTAL

The kinetic studies on the degradation of streptozotocin in aqueous solution were conducted with the use of three assay procedures.

**Kinetics by Color Assay.**—One assay procedure used was a color assay developed by Forist (7) and based on the acid cleavage of the N-nitroso group of streptozotocin to yield nitrous acid, subsequently diazotized with sulfanilic acid, and the salt coupled with N(1-naphthyl)-ethylenediamine to produce an azo compound with a stable absorption maximum of reproducible absorbance at 550  $m\mu$  after subjection to 60° for forty-five minutes.

The streptozotocin was prepared by Herr, *et al.*, (3); the characterization of this material has been reported (3). The antibiotic was dissolved in acid, acetate, and phosphate buffers as given in Tables I

and II and maintained at the appropriate temperature. The buffer solutions had been previously equilibrated at that temperature. The initial concentrations of the solutions were 0.22 mg./ml. except for runs 8 and 10 which were 0.44 mg./ml. At recorded time intervals 1.00-ml. aliquots of the buffered solutions were pipetted and each was mixed with 10 ml. of pH 4.00 acetate buffer. One milliliter of the resultant solution was pipetted into 5 ml. of the combined color reagent and the color developed at 60° for forty-five minutes. This solution was cooled to room temperature and the absorbance was read at 550  $m\mu$  on the Beckman model B. The original material was also assayed to determine the color proportional to the original concentration. Adherence to Beer's law had been previously confirmed (7). A spectrophotometric blank omitting the antibiotic was prepared similarly for each assay.

**Polarographic Assay.**—It had been observed<sup>1</sup> that streptozotocin possessed a polarographic wave of  $E_{1/2} = ca. -0.8$  v. in pH 4.6 acetate buffer. The magnitude or  $i_d$  of this wave can be correlated with streptozotocin concentration as at pH 4 in acetate buffer (see Fig. 1).

The general procedure for polarographic assay was to purge all buffer solutions with nitrogen in excess of thirty minutes and equilibrate them at the desired temperature of the study. The weighed samples of streptozotocin were brought up to volume with the desired buffer. The final buffer concentrations are listed in Tables II and III. The concentrations in

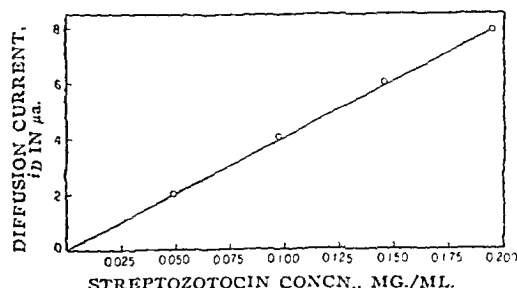


Fig. 1.—Polarographic calibration curve; diffusion current,  $i_d$ , in  $\mu$ A. against streptozotocin concentration in mg./ml. for acetate buffer, pH 4.04.

<sup>1</sup> By Dr. E. C. Olson of these laboratories

\* Received February 23, 1960, from the Research Division, The Upjohn Co., Kalamazoo, Mich.

The author is greatly indebted to Mrs. Lillian G. Snyder for excellent technical assistance, to Drs. W. T. Sokolski and L. J. Hanka, Mr. M. R. Burch and associates for the microbiological assays, and to Mr. D. J. Weber for the potentiometric titrations.

TABLE I—CONDITIONS AND RATE CONSTANTS ( $k$  IN SEC<sup>-1</sup>) FOR THE APPARENT FIRST-ORDER DEGRADATION OF STREPTOZOTOCIN<sup>a</sup> AT 30.2° AS DETERMINED BY COLOR ASSAY (7)

Run No	pH	Buffer Composition		Exptl 10 <sup>5</sup> $k$ (sec <sup>-1</sup> )
		[HCl]		
1	1 21	0 075		10 6
2	1 48	0 04		7 41
3	1 74	0 02		4 89
4	2 02	0 01		3 15
		[CH <sub>3</sub> COOH]	[CH <sub>3</sub> COO <sup>-</sup> ] = $\mu$	
5	3 40	0 1756	0 0122	1 25
6	3 96	0 1334	0 0333	0 694
7	4 60	0 025	0 025	
8	4 59	0 100	0 100	0 959
9	4 58	0 200	0 200	
10	4 61	0 400	0 400	
11	4 60	0 025	0 025	
12	4 96	0 0326	0 0837	1 40 <sup>b</sup>
13	5 84	0 0040	0 098	1 12
		[H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ]	[HPO <sub>4</sub> <sup>-</sup> ]	
14	6 89	0 0505	0 0758	5 05 <sup>c</sup>
15	7 32	0 0225	0 0901	44 6 <sup>c</sup>
16	7 69	0 0107	0 0961	86 9 <sup>c</sup>
17	8 00	0 0041	0 0994	150 <sup>c</sup>
				268 <sup>c</sup>

<sup>a</sup> 0.22 mg/ml except for runs 8 and 10 of 0.44 mg/ml<sup>b</sup> This solution when corrected for absorbance at 550 m $\mu$  prior to heating gave a rate constant of  $2.06 \times 10^{-5}$ <sup>c</sup> The run no., ionic strength,  $\mu = [\text{H}_2\text{PO}_4^-] + 2.5 [\text{HPO}_4^-]$ , and calculated rate constant  $10^5 k$  (sec<sup>-1</sup>) where  $k = 33 \times 10^{-5} ([\text{HPO}_4^-] + 158 [\text{OH}^-] + 0.20 \times 10^{-5})$  where  $[\text{OH}^-] = 10^{-\text{pOH}} = 10^{-(\text{pH} - \text{pH}_0)} = 10^{-(13.83 - \text{pH})}$  are 13, 0.098, 3.7, 14, 0.2490, 45.2, 15, 0.2478, 80.5, 16, 0.2510, 149, and 17, 0.2526, 269TABLE II—CONDITIONS AND RATE CONSTANTS ( $k$  IN SEC<sup>-1</sup>) FOR THE APPARENT FIRST-ORDER DEGRADATION OF STREPTOZOTOCIN<sup>a</sup> AT 30.2° AS DETERMINED BY POLAROGRAPHIC ASSAY

Run No	pH	Buffer Composition		10 <sup>5</sup> $k$ (sec <sup>-1</sup> )	
		[HCl]		Exptl	Calcd <sup>b</sup>
18	0 32 (0 42) <sup>c</sup>	0 50		22 3	21 1
19	0 43 (0 52) <sup>c</sup>	0 40		19 2	19 1
20	0 51 (0 64) <sup>c</sup>	0 30		16 7	16 9
21	0 70 (0 82) <sup>c</sup>	0 20		14 4	14 4
22	1 21 (1 21) <sup>c</sup>	0 075		10 1	10 0
23	1 48 (1 47) <sup>c</sup>	0 04		7 68	7 68
24	1 74 (1 75) <sup>c</sup>	0 02		5 56	5 59
25	2 02 (2 03) <sup>c</sup>	0 01		4 10	4 17
		[CH <sub>3</sub> COOH]	[CH <sub>3</sub> COO <sup>-</sup> ]		
26	3 42	0 1756	0 0122	2 01	2 14
27	3 98	0 1334	0 0333	1 71	2 03
28	4 60	0 025	0 025	2 27	2 10
29	4 60	0 400	0 400	2 42	2 10
30	4 96	0 1163	0 0837	2 44	2 19
31	5 86	0 1020	0 098	6 11	3 70

<sup>a</sup> 0.60 mg/ml except for runs 28 and 29 of 0.22 mg/ml<sup>b</sup> Calculated from  $k = k_2[\text{H}^+]/(1 + [\text{H}^+]/K_a) + k_1[\text{H}^+]/(1 + K_a/[\text{H}^+]) + k_0$  where  $k_2 = 2.76 \times 10^{-4}$ ,  $k_1 = 2.38 \times 10^{-5}$ ,  $K_a = 4.51 \times 10^{-7}$ ,  $k_0 = 2.00 \times 10^{-6}$  where  $[\text{H}^+] = f_{\text{HCl}}[\text{HCl}]$ <sup>c</sup> pH values in parentheses are determined from the mean activity coefficients,  $f_{\text{HCl}}$  of HCl in water (9a) at 30° by pH-log  $f_{\text{HCl}}[\text{HCl}]$ 

all cases (except for runs 28 and 29 which were 0.22 mg/ml) were 0.60 mg/ml. An aliquot of 8.00 ml was removed from the degrading solution and diluted up to 25 ml with previously nitrogen-purged 0.1 M sodium acetate-acetic acid except for runs 24 and 25 which were run without dilution. These final aliquots were purged with nitrogen for ten minutes and a polarogram taken at 25° on the Leeds and Northrup recording electrochemograph against a saturated calomel electrode. Blank solutions, omitting the antibiotic, were treated similarly each day.

The acetate buffer concentration has an effect on the half wave potential,  $E^{1/2}$ , and the diffusion current,  $i_D$ . The data from the polarograph studies are plotted in Fig 2. However, at a given acetate buffer concentration, the  $i_D/C$  and  $E^{1/2}$  for streptozotocin show no significant variation with the concentration,  $C$ , of the antibiotic.

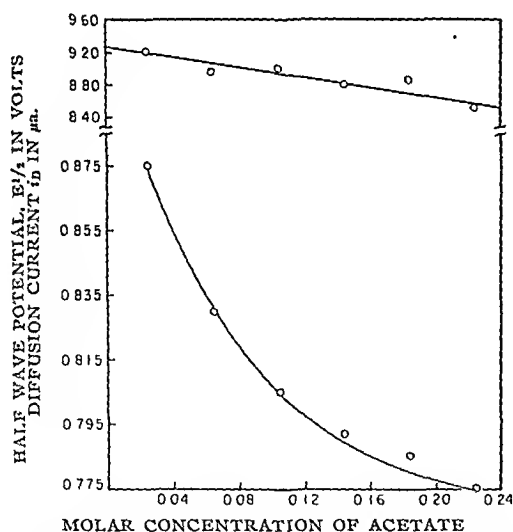
Correlation of Color and Polarographic Assay with *Proteus vulgaris* Bioassay by the Disk-Plate Method.

Simultaneously with the color assays of streptozotocin degradation at pH 4.6, run No 8, Table 1, bioassays were run against *P. vulgaris* by the disk-plate method. Similarly, bioassays were run simultaneously with the polarographic studies of runs 25, 26, and 31, Table II.

The general procedure was to dilute a 1.00-ml aliquot of the streptozotocin to be assayed with pH 4 phosphate solution (0.1 M) to the concentration range suitable to the disk-plate assay. Aliquots taken were so treated and refrigerated until 4 p.m. when all samples were bioassayed.

TABLE III.—CONDITIONS AND RATE CONSTANTS ( $k$  IN SEC.<sup>-1</sup>) FOR THE APPARENT FIRST-ORDER DEGRADATION OF STREPTOZOTOCIN AT VARIOUS TEMPERATURES

Run No.	pH	°C.	10 $k$	Buffer Composition	
				[H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ]	[HPO <sub>4</sub> <sup>-</sup> ]
14	6.89	30.2	4.46 <sup>a</sup>	0.0513	0.075
32	6.90	40.0	18.4 <sup>a</sup>	0.0513	0.075
33	6.89	50.2	73.2 <sup>a</sup>	0.0513	0.075
34	6.90	60.5	268 <sup>a</sup>	0.0513	0.075
35	7.31	40.0	37.2 <sup>a</sup>	0.0225	0.0901
36	7.72	40.0	70.9 <sup>a</sup>	0.0107	0.0961
37	8.08	40.0	163 <sup>a</sup>	0.0041	0.0994
28	4.60	30.2	0.227 <sup>b</sup>	0.025	0.025
38	4.67	39.4	0.313 <sup>b</sup>	0.025	0.025
39	4.62	50.2	1.48 <sup>b</sup>	0.025	0.025
40	4.65	60.0	3.58 <sup>b</sup>	0.025	0.025
41	4.65	69.5	12.0 <sup>b</sup>	0.025	0.025
25	2.02	30.2	0.410 <sup>c</sup>	0.01	
42	2.02	39.4	0.985 <sup>c</sup>	0.01	
43	2.23	50.2	3.13 <sup>c</sup>	0.01	
44	2.24	59.7	7.89 <sup>c</sup>	0.01	
45	1.92	69	19.1 <sup>c</sup>	0.01	
46	1.76	40.0	1.96 <sup>c</sup>	0.02	
47	1.48	40.0	2.59 <sup>c</sup>	0.04	
48	1.22	40.0	3.28 <sup>c</sup>	0.075	

<sup>a</sup> By color assay.<sup>b</sup> By polarographic assay, 10 $k$  values by corrective color assay (uncorr. in parentheses) for each of these runs are 28: 0.206 (0.141); 38: 0.265 (0.221); 39: 1.46 (1.28); 40: 3.49 (3.49); 41: 11.5 (11.5).<sup>c</sup> By polarographic assay.Fig. 2.—Effect of acetate ion concentration on the  $E_{1/2}$  and  $i_d$  of streptozotocin polarography. The pH is 4.6, the streptozotocin is 0.22 mg./ml.

The standard used had an assigned value of  $180 \pm 20 \mu\text{g.}$

**Correction for Color Artifact.**—The apparent discrepancy in estimated rate constants from color and polarographic assay is clearly shown by comparison of runs 1–13, Table I, and runs 22–31, Table II. The discrepancy is most apparent in the 2.0–5.0 pH range at 30°. This can be explained by a positive interference in the color assay as the degradation proceeds. Forist (7) has pointed out that nitrite could be a product of degradation and give an apparent assay for intact antibiotic.

Nitrite most probably would be a degradation product in the acidic range. Addition of color reagent developed color at room temperature at pH values less than 6. No such appearance at room temperature was observed above pH 6. Naturally, such an artifact would give lower estimates for rate constants. However, compensation for such an artifact could be made by measuring the color due to nitrite and subtracting from the total. Higher temperature or higher acidities would tend to decompose the nitrite interfering in the color assay, probably to nitric oxide and nitric acid.

In another procedure, the color absorbance was read immediately at 550 m $\mu$  as a function of time at room temperature. Color developed instantaneously and then enhanced slowly in the cold and the extrapolated intercept at zero time was assumed to be the amount of color corresponding to the nitrite artifact. Examples are given in Fig. 3. This intercept value, which increased with the time of degradation, is consistent with the premise of nitrite as a product of degradation. This intercept value when subtracted from the total value obtained from the normal color assay procedure gave a net absorbance which was considered proportional to the streptozotocin content.

The plots of Fig. 3 can be interpreted as demonstrating the following facts: (a) There is initially no nitrite present. (b) Nitrite appears as the degradation proceeds and although it reacts with reagent quickly to form color, the reaction is not instantaneous. The total time of color formation with nitrite is up to eight minutes. (c) The production of color at room temperature with the streptozotocin is a slower process than with the nitrite, but it is significant and correction for interfering streptozotocin color is necessary. The



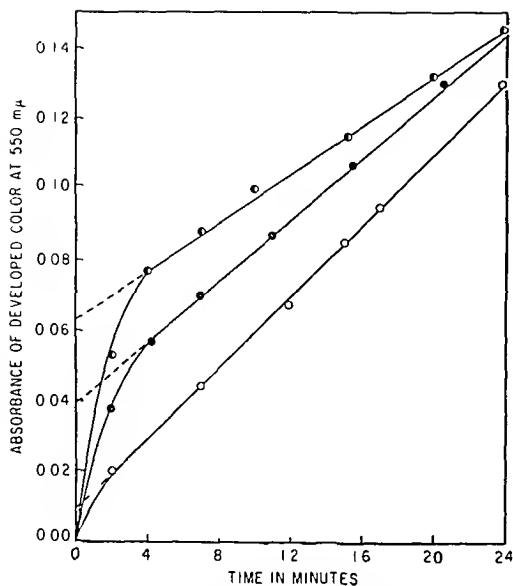


Fig 3 — Color development at 550  $m\mu$  in the cold as a function of time from streptozotocin (0.22 mg/ml) aliquots degraded in 0.025  $M$  acetate buffer, pH 4.6 at 30.2°. The symbols are for aliquots degraded at different times in hours: ○, 23.5; ◐, 46.9; and ●, 97.0

extrapolation procedure should be valid as the streptozotocin color formation simulates pseudo zero order. The slopes of these straight lines are as expected, decreasing with lower concentrations of nondegraded streptozotocin.

The first order plots of log color assay *versus* time for run 11, Table I, uncorrected and corrected for this initial color development in the cold are given in Fig 4 where it can be seen that the rate based on the corrected assays is significantly greater. The rate based on the corrected color assay is indistinguishable from that based on the polarographic assay, run 28, Table II.

**Potentiometric Titrations.**—The following studies were replicated. The parenthetical values report the results of the second series.

Streptozotocin, 20 mg, *ca* 0.044 mmoles, mol wt 457 (3), was dissolved in 20 ml of 0.085  $M$  hydrochloric acid and allowed to stand for twenty-four hours, *ca* two half lives, and subsequently titrated with 1  $M$  sodium hydroxide. A grouping of  $pK_a$  *ca* 10.8 was the only titratable functionality observed and was in the amount comparable to or greater than the initial mmoles of streptozotocin, 0.045 mmoles, in both replications. This material stood for a short time under nitrogen at high alkalinity, apparent pH *ca* 12, and was back titrated with 1  $M$  hydrochloric acid. The functionality of  $pK_a > 10$  was still present. Also a small quantity of a functionality of  $pK_a$  6.2 appeared, 0.015 mmoles (0.020 mmoles) indicative of bicarbonate and confirming the observations of Herr, *et al* (3).

Streptozotocin, 20 mg, *ca* 0.044 mmoles, was dissolved in 20 ml 0.0902  $M$  sodium hydroxide and titrated with 1  $M$  hydrochloric acid. Functional groups of  $pK_a$  10–11, estimated as greater

in quantity than the initial amount of antibiotic, were observed. In addition, amounts of functionalities of  $pK_a$  9.2, 0.025 mmoles (0.020 mmoles) and  $pK_a$  6.2, 0.043 mmoles (0.045 mmoles) were observed. On back titration with 1  $M$  sodium hydroxide the functionality at 6.1  $pK_a$  disappeared while the 0.025 mmoles (0.020 mmoles) of  $pK_a$  9.2 remained as did functionality *ca*  $pK_a$  10.8.

## CALCULATIONS AND RESULTS

**Empirical Equations for Degradation.**—The data for streptozotocin assay as a function of time for constant pH conditions can be fitted as a first order plot according to the expression

$$\log X = -(k/2.303)t + \log X_0 \quad (\text{Eq 1})$$

where  $X$  is either the absorbance,  $A$ , at 550  $m\mu$  from the color assay or the diffusion current,  $i_d$ , in  $\mu A$ , at  $-1.05$  v from the height of the polarographic wave, where  $X_0$  is the pertinent value at time zero.

Typical first-order plots of streptozotocin degradation based on the color assay are given in Figs 4, 5, and 6. The estimated rate constants,  $k$  in  $\text{sec}^{-1}$ , for these and other studies at 30.2° are given in Table I.

Typical first order plots by the polarographic assay are given in Fig 7. The estimated rate constants,  $k$  in  $\text{sec}^{-1}$ , for these and other studies at 30.2° are given in Table II.

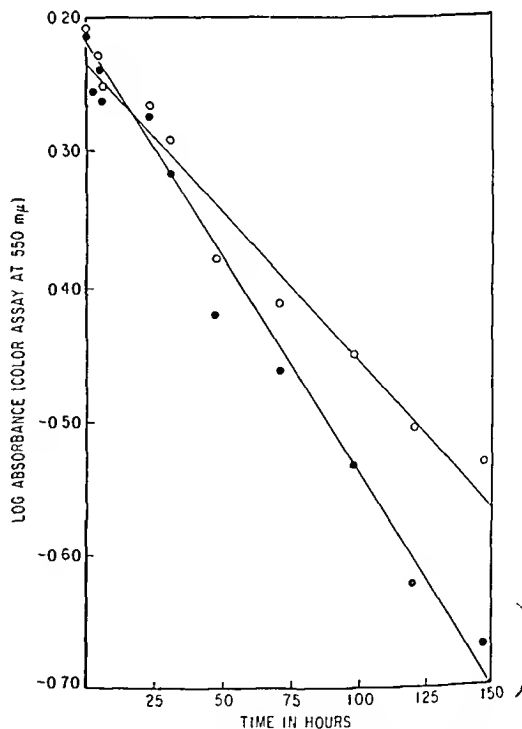


Fig 4 — Effect of nitrite contaminant on apparent first order plots of streptozotocin degradation at 30° in 0.025  $M$  acetate buffer, pH 4.6: ○ by normal procedure; ● after correction for room temperature color development.

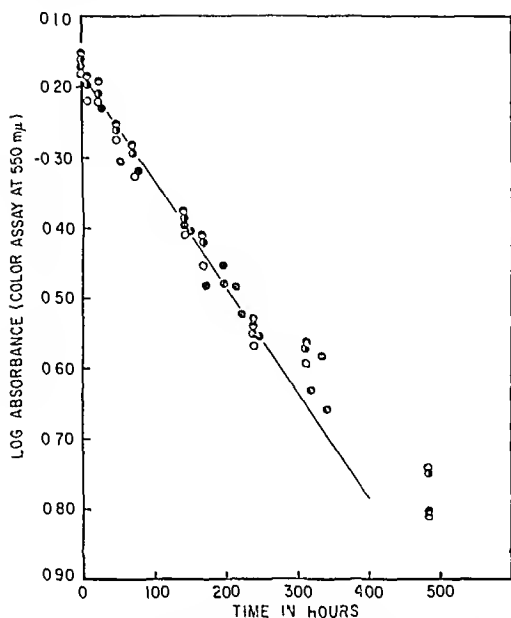


Fig 5—Apparent first-order plots of streptozotocin degradation at 30° at constant pH but with varying acetate ion concentration, pH = 4.6, as determined by color assay

Code	[HOOCCCH <sub>3</sub> ]	[ <sup>-</sup> OOCCH <sub>3</sub> ]	Run
○	0.025	0.025	7
●	0.100	0.100	8
◐	0.200	0.200	9
◑	0.400	0.400	10

Varying acetate concentrations at constant pH had no significant effect on the rate constant. Figure 5 shows the nonsignificant effect of varying acetate ion concentrations from 0.025 to 0.400 M at pH 4.6 on the first-order plots based on color assay, i.e., runs 7 through 10 of Table I. Also see runs 28 and 29 at two different acetate concentrations in Table II.

The values for the rate constants derived from color assay are lower than those derived from the polarographic assays below pH 6. This fact is attributed to the production of color from the nitrite product of acid degradation which gives rise to an erroneously high value for intact antibiotic. At high acidities nitrite becomes readily degraded, at extremely low acidities none is probably formed, and in the intermediate pH regions, ca 2–6, the nitrite ion remains to interfere.

The logarithmic values of the apparent first-order rate constants at 30° as  $\log k$  ( $k$  in  $\text{sec}^{-1}$ ) are plotted against the pH in Fig 8. The solid circles are based on the rate constants determined from the color assays on studies above pH 5.5; the open circles are based on the rate constants determined from the polarographic assays on studies below pH 6.0.

Empirical relations that can express apparent first-order rate constant dependence on pH and reasonably fit the plotted data of Fig 8 at 30° are

$$\log k = -0.453 \text{ pH} - 4.445, \text{ pH} < 2.5 \quad (\text{Eq } 2)$$

$$\log k = 0.73 \text{ pH} - 9.42, \text{ pH} > 6.0 \quad (\text{Eq } 3)$$

However, since  $k_{300} \sim 2 \times 10^{-6} \text{ sec}^{-1}$  when  $2.5 < \text{pH} < 5.5$ , the  $k$  values derived from Eqs 2 and 3 do not account for the degradation rate in the pH range 2.5 to 5.5.

**Degradation at Acidic pH Values.**—Streptozotocin has no apparent pKa values by potentiometric titration (3). However, a rational explanation for the apparent fractional slope of the  $\log k$  versus pH plot at 30° in the acid region (see Fig 8) as given in the empirical Eq 2 is that we are actually measuring a transition region of hydrogen ion attack on protonated,  $\text{SH}^+$ , and nonprotonated streptozotocin, S, in ionic equilibrium with each other.

Actually, the rate of total streptozotocin,  $[\text{S}]$ , concentration change as catalyzed by hydrogen ion could be the sum of the rates of change of the protonated,  $d[\text{SH}^+]/dt$ , and nonprotonated  $d[\text{S}]/dt$ , forms so that

$$\begin{aligned} d[\text{S}]/dt &= d([\text{S}] + [\text{SH}^+])/dt \\ &= -k_1[\text{H}^+][\text{SH}^+] - k_2[\text{H}^+][\text{S}] \\ &= -k_{\text{H}^+}[\text{H}^+][\text{SH}^+] + [\text{S}] \\ &= -(k - k_0)[\text{S}] \end{aligned} \quad (\text{Eq } 4)$$

where  $k$  is the apparent first order rate constant at an acidic pH and  $k_0 \sim 2.0 \times 10^{-6} \text{ sec}^{-1}$  is the apparent first order, pH independent, rate constant from the data of Table II, runs 26 through 30.

The ionization equilibrium



may be formulated as

$$[\text{S}]/[\text{SH}^+] = K_a/[\text{H}^+] \quad (\text{Eq } 6)$$

where  $K_a$  is the ionization constant of the equilibrium given in Eq 5. By a method analogous to that of Edwards (8) it can be shown that

$$(k - k_0)/[\text{H}^+] = k_{\text{H}^+} = k_1/(1 + K_a/[\text{H}^+]) + k_2/(1 + [\text{H}^+]/K_a) \quad (\text{Eq } 7)$$

Also, from the relationships of Eqs 4 and 7, it can be shown that

$$k - k_0 = (k_2 - k_{\text{H}^+})K_a + k_1[\text{H}^+] \quad (\text{Eq } 8)$$

At some other hydrogen ion concentration,  $[\text{H}^+]',$  the apparent first-order rate constant is  $k'$  where

$$(k' - k_0)/[\text{H}^+]' = k'_{\text{H}^+} \quad (\text{Eq } 9)$$

$$k' - k_0 = [k_2 - k'_{\text{H}^+}]K_a + k_1[\text{H}^+]' \quad (\text{Eq } 10)$$

Subtraction of Eq 10 from Eq 8 and rearrangement results in

$$\frac{(k - k')}{[\text{H}^+] - [\text{H}^+]' } = \frac{(k'_{\text{H}^+} - k_{\text{H}^+})}{[\text{H}^+] - [\text{H}^+]' } K_a + k_1 \quad (\text{Eq } 11)$$

It follows that from any two studies  $k - k', [\text{H}^+] - [\text{H}^+]',$  and  $k'_{\text{H}^+} - k_{\text{H}^+} = (k' - k_0)/[\text{H}^+]' - (k - k_0)/[\text{H}^+]$  may be calculated. The hydrogen ion activity is used for the  $[\text{H}^+]$  values and is considered as

$$a_{\text{H}^+} = [\text{H}^+] = 10^{-\text{pH}} \quad (\text{Eq } 12)$$

However, since the pH measurement is inaccurate at low pH values, the hydrogen ion activities are determined from

$$a_{\text{H}^+} = f_{\text{HCl}}[\text{HCl}] \quad (\text{Eq } 13)$$

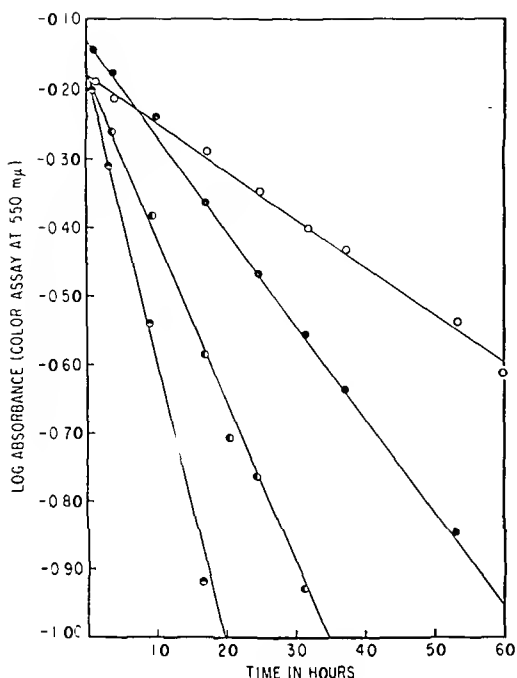


Fig. 6.—Apparent first-order plots of streptozotocin degradation at 30° in phosphate buffers as determined by color assay.

Code	pH	Run
○	6.89	14
●	7.32	15
●	7.69	16
●	8.00	17

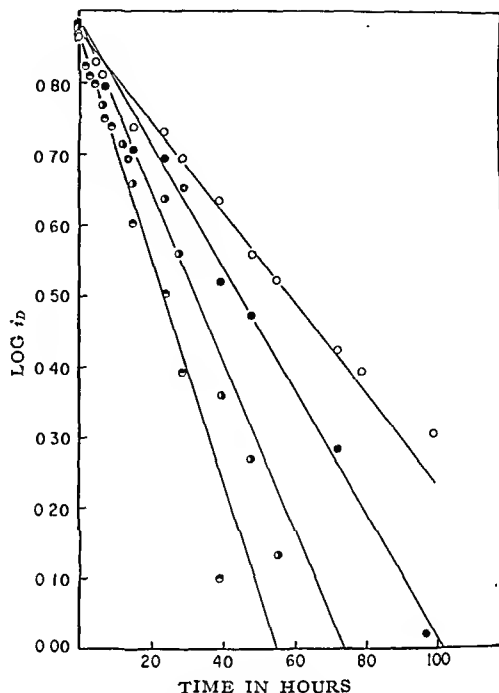


Fig. 7.—Apparent first-order plots of streptozotocin degradation at 30° in hydrochloric acid as determined by polarography where  $i_D$  is taken at  $E_{1/2} = -1.05$  v.

Code	HCl	pH	Run
○	0.010	2.02	25
●	0.020	1.74	24
●	0.040	1.49	23
●	0.075	1.22	22

as based on the literature values of the activity coefficient (9a),  $f_{\text{HCl}}$ , and the actual HCl concentrations. Experimental and actual pH ( $= -\log f_{\text{HCl}}[\text{HCl}]$ ) values are given in Table II for the actual concentrations of HCl used. It is observed that above pH 1.0 the agreement between calculated and experimental pH values is good.

When runs 18 through 25, Table II, are paired in all possible combinations, the data permit the calculation of the functions,  $(k - k')/([H^+] - [H^+])'$  and  $(k'_{\text{H}^+} - k_{\text{H}^+})/([H^+] - [H^+])'$  for each pair. When the former is plotted against the latter to validate the postulates behind Eq. 11, a straight line should result with slope  $K_a$  and intercept  $k_1$ . Such a plot of paired values is given in Fig. 9. The numbers at each plotted point refer to the paired runs listed in Table II for which the functions were calculated.

The intercept,  $k_1$ , of Fig. 9 is  $2.38 \times 10^{-5}$  1/molc/sec. and the slope,  $K_a$ , is  $4.51 \times 10^{-2}$  or  $\text{p}K_a = 1.35$  at 30°. Such a  $\text{p}K_a$  would not be observed on classical potentiometric titration (3). The  $k_2$  value calculated from Eq. 8 for runs 18 through 25, Table II, is  $2.76 \times 10^{-4}$  1/M/sec.

Thus Eq. 7 at 30° becomes

$$k_{\text{H}^+} = 2.38 \times 10^{-5} / (1 + 4.51 \times 10^{-2} / [H^+]) + 2.76 \times 10^{-4} / (1 + [H^+] / 4.51 \times 10^{-2}) \quad (\text{Eq. 14})$$

Use of this equation permits the calculation of

apparent first-order rate constants at 30° by

$$k = k_{\text{H}^+}[H^+] + k_0 \quad (\text{Eq. 15})$$

where  $[H^+]$  is defined by Eq. 13. The rate constants calculated on this basis for the pH range 0 to 5 are given in Table II and demonstrate agreement with the experimental values. The drawn solid curve in Fig. 8 for the acid branch is the calculated curve; the plotted points are the experimental values where pH is taken as  $-\log f_{\text{HCl}}[\text{HCl}]$ . Agreement is observed.

**Degradation at Alkaline pH Values.**—A similar rationale is not applicable to explain the apparent fractional slope of the  $\log k$  versus pH plot at 30° in the alkaline region (see Fig. 8) as given in the empirical Eq. 3. A set of simultaneous equations can be postulated however. They are

$$k = k_{\text{H}_2\text{PO}_4^-} [\text{H}_2\text{PO}_4^-] + k_{\text{HPO}_4^{2-}} [\text{HPO}_4^{2-}] + k_{\text{OH}^-} [\text{OH}^-] + k_0 \quad (\text{Eq. 16})$$

where the  $k$  values, pH values, and  $[\text{H}_2\text{PO}_4^-]$  and  $[\text{HPO}_4^{2-}]$  concentrations are given in Table I, runs 14–17. The  $[\text{OH}^-]$  concentration can be calculated from

$$[\text{OH}^-] = 10^{-\text{pOH}} = 10^{-(\text{p}K_w - \text{pH})} \quad (\text{Eq. 17})$$

where  $\text{p}K_w$  is 13.83 at 30° (9b).

The  $k_{\text{H}_2\text{PO}_4^-}$  value is negligible and on its omission

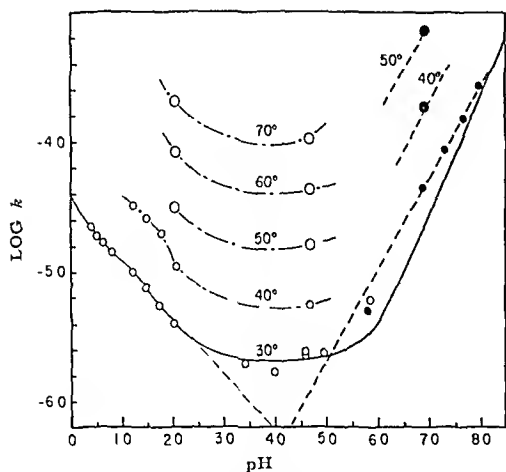


Fig. 8.—pH profile of the logarithm of the apparent first-order rate constant,  $k$  in  $\text{sec.}^{-1}$ , for the hydrolytic degradation of streptozotocin at several temperatures. The dashed lines are empirical relations for acidic and alkaline hydrolysis. The solid curve at  $30^\circ$  is based on a streptozotocin ionic equilibrium in the range and ignores possible monohydrogen phosphate ion catalysis in the alkaline range, i.e., considers hydroxyl ion and water catalyzed degradation only. The dot-dash curves estimate the log  $k$ -pH profile at other temperatures. The open circles are by polarographic assay; the closed circles are by color assay.

from Eq. 16, the estimated values are  $k_{\text{OH}^-} = 158 \text{ L./mole/sec.}$ ,  $k_{\text{HPO}_4^-} = 33 \times 10^{-5} \text{ L./mole/sec.}$  where  $k_0$  is  $0.20 \times 10^{-5} \text{ sec.}^{-1}$ .

This result implies significant catalytic degradation by monohydrogen phosphate ion. Use of these parameters in Eq. 16 where  $k_{\text{HPO}_4^-} = 0$  gives calculated values in good agreement with the experimental (see runs 14 through 17, Table I).

The variation in rate from strict hydroxyl ion catalysis cannot be ascribed to ionic strength,  $\mu$ . An expression for  $\mu$  simplifies to

$$\mu = [\text{H}_2\text{PO}_4^-] + 2.5[\text{HPO}_4^-] \quad (\text{Eq. 18})$$

and shows very little variation from 0.25 among runs 14 through 17, Table I.

The drawn solid curve in Fig. 8 is not intended to agree with the plotted experimental points of the alkaline branch since it is based solely on the "spontaneous" rate,  $k_0$ , and the specific hydroxyl ion catalysis, i. e.

$$k = k_0 + k_{\text{OH}^-} [\text{OH}^-] \quad (\text{Eq. 19})$$

**Degradation at Several Temperatures.**—Table II provides the conditions and rate constants ( $k$  in  $\text{sec.}^{-1}$ ) for the apparent first-order degradation of streptozotocin at various other temperatures. These values are also plotted in Fig. 8 with the dot-dashed lines estimates of the log  $k$  versus pH functions at a given temperature.

The Arrhenius plots for the data of pH 2.03, 4.65, and 6.90 are given in Fig. 10. The parameters of the Arrhenius equation

$$\log k = -S_1/T + \log P \\ = -\Delta H_a/2.303RT + \log P \quad (\text{Eq. 20})$$

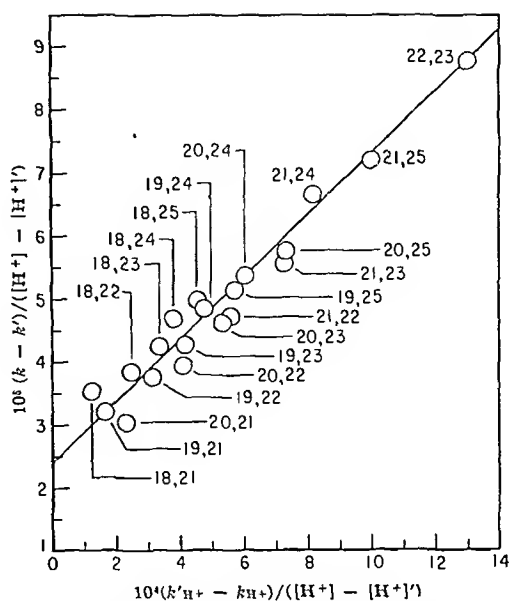


Fig. 9.—Plot of the linearly related functions to permit estimates of the slope, i.e., the dissociation constant,  $K_a$ , of protonated streptozotocin, and the intercept, i.e., the bimolecular rate constant,  $k_1$ , for the hydrogen ion catalyzed degradation of this protonated streptozotocin. The numbers pertinent to the data points represent the paired studies of Table II used in the calculation of the functions.

are defined and given in Table IV. The Arrhenius plot for the 4.65 pH data has greater variation than the others. Nevertheless, the estimated slope for the 4.65 pH is not inconsistent with that for the 2.03 data. The plotted data in Fig. 8 for temperatures other than  $30^\circ$  are based on the curves drawn for the Arrhenius relations in Fig. 10.

**Correlations of Bioassay and Chemical Assays.**—The possible correlations of the bioassay (4) with color assays (7) are plotted in Fig. 11, of the bioassay with polarographic assay are plotted in Figs. 12, 13, and 14 for degradation at several pH values. The statistics for the least squares plots are given in Table V.

Although the bioassay ( $y$ ) is considered as the dependent and the chemical assay ( $x$ ) as the independent (or error-less) variable in the statistics of Table IV, the plots in the figures are of  $x$  versus  $y$ .

Inspection of the table and figures permits the conclusion that only the polarographic assays at degradations of pH 3.42 and 5.86 can be correlated with the bioassay, where slopes and intercepts are not significantly different from the theoretical 1.0 and 0.0. Figures 12 and 13 show that the agreement would be even more coincident if there had not been one or two extreme values at the higher concentrations. However, this is not too serious as a mild difference in slope from unity but no difference in intercept from zero merely signifies that the physicochemical factor (absorbance or diffusion current) chosen to represent a unit concentration may have been based on a sample of slightly greater streptozotocin content than that of the bioassay standard. Of greater importance is

TABLE IV — APPARENT THERMODYNAMIC QUANTITIES<sup>a</sup> FOR THE HYDROLYTIC DEGRADATION OF STREPTOZOTOCIN

Apparent pH	S	ΔH <sub>a</sub>	log P	Buffer Composition:
6.90	5,921	27.1	15.19	[H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> ] = 0.051, [HPO <sub>4</sub> <sup>-</sup> ] = 0.0750
4.65	4,500	20.6	9.19	[CH <sub>3</sub> COOH] = 0.025, [CH <sub>3</sub> COO <sup>-</sup> ] = 0.025
2.03	4,500	20.6	9.41	[HCl] = 0.010

<sup>a</sup> The quantities are derived from the logarithmic form of the Arrhenius relation  $\log k = -(\Delta H_a/2.303R)(1/T) + \log P = -5/T + \log P$  where the  $k$  is in  $\text{sec}^{-1}$ , the  $\Delta H_a$  is the heat of activation in kcal/mole,  $R$  is the gas constant in cal/degree/mole,  $T$  is the absolute temperature, and  $\log P$  is a constant associated with entropy.

TABLE V — STATISTICS OF REGRESSIONS ( $y = mx + b$ ) OF BIOASSAYS ( $y$ ) ON CHEMICAL ASSAYS ( $x$ )

Assay	30° pH	Degradation Run	Fig	No. of Assays	Least Squares Fit	$\sigma_b$	$\sigma_m$	$\sigma_y$ , mg/ml	Remarks
Color	4.6	8	11	19	$y = 1.424x - 124.6$	54.3	0.219	76.1	Cannot assume $y = 0$
Polarograph	3.42	26	12	11	$y = 1.242x - 74.2$	58.5	0.148	85.9	The slope, $m$ , is not significantly different than 1.0; the intercept, $b$ , is not significantly different than 0. For $b = 0$ , $m = 1.074$ ; $\sigma_m = 0.068$
Polarograph	5.86	31	13	8	$y = 1.311x - 56.9$	50.5	0.131	65.8	As above, except when $b = 0$ , $m = 1.180$ , $\sigma_m = 0.062$ , and $m$ is significantly different from 1.0
Polarograph	2.02	25	14	13	$y = 1.226x - 134.8$	33.0	0.086	48.5	Both slope, $m$ , and intercept, $b$ , are significantly different from the theoretical values 1.0 and 0.0

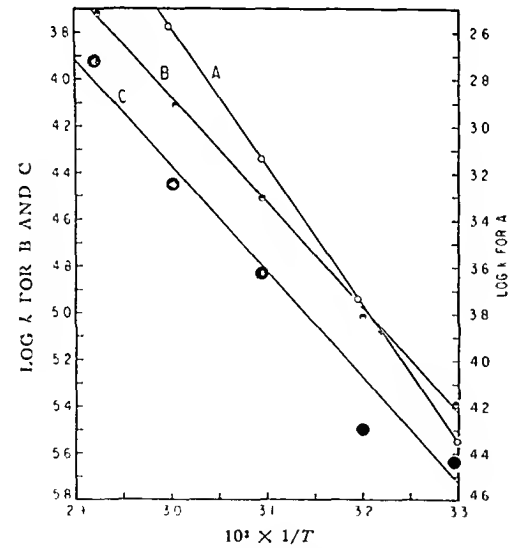


Fig. 10 — Arrhenius plots for streptozotocin degradation at several pH values: curve A, pH 6.90 in phosphate buffers; curve B, pH 4.65 in acetate buffers; and curve C, pH 2.03 in hydrochloride buffers

that the intercept should not be significantly non-zero. The significant deviation of the intercept from zero is readily explained in the case of the color assay (degradation at pH 4.6, Fig. 11) by the appearance of an artifact, nitrite, as the degradation

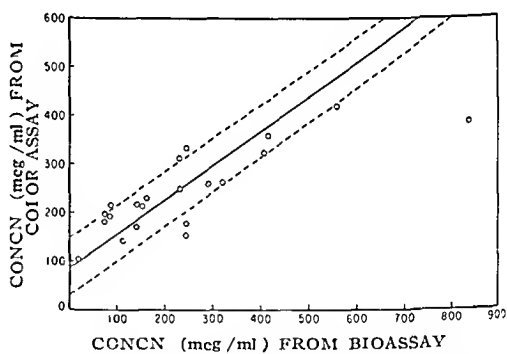


Fig. 11 — Attempted correlation of streptozotocin color assay with bioassay on material degraded at 30°, pH = 4.6 (run No. 8). The solid line is fitted by least squares; the dashed lines represent the standard deviation of bioassay about regression

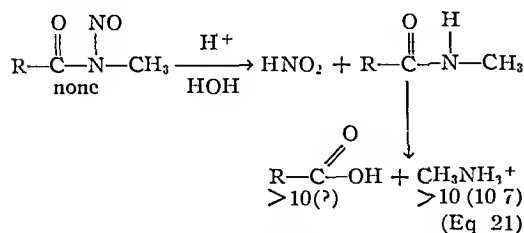
proceeded, which produced color construed to be antibiotic. Thus when the material had degraded to zero bioassay, the color assay might still indicate streptozotocin. In the case of the degradation at pH 2.02, Fig. 14, the significantly nonzero intercept implies acid degradation of the antibiotic at some other site than the N-nitrosoamide grouping

### DISCUSSION

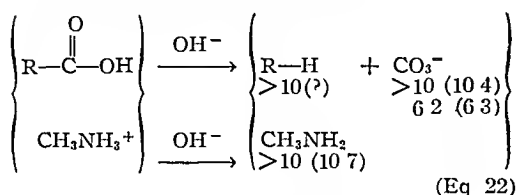
**Mechanism of Streptozotocin Degradation.**—The following scheme summarizes the evidence on the degradation of streptozotocin (1) and presents a possible mechanism. The values under the com-

pounds represent the assignment of observed pKa values. The parenthetical value is the handbook pKa. The symbol (?) represents doubtful assignments of experimental pKa values.

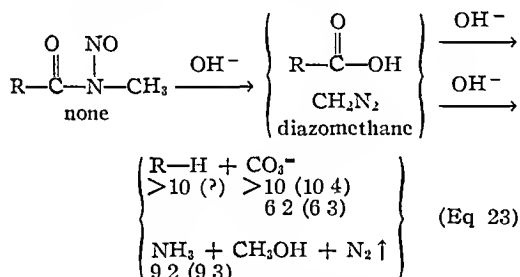
#### Acid degradation of streptozotocin:



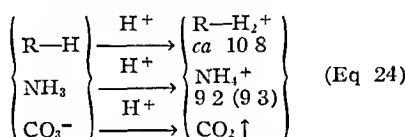
#### Alkaline digestion of acid degraded streptozotocin:



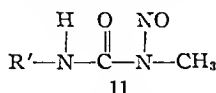
#### Alkaline degradation of streptozotocin:



#### Acid digestion of alkaline degraded streptozotocin



Since the R-H is the only structure that can be assigned the pKa ca 10.8 observed on the alkaline titration after the acid digestion of the alkaline degraded streptozotocin, it follows that R-H<sub>2</sub><sup>+</sup> may actually be R'-NH<sub>3</sub><sup>+</sup> and it is possible that the N-nitrosomethylamide group is actually a substituted N-nitrosomethylurea.<sup>2</sup>



The N-nitrosomethylamide (or N-nitrosomethylurea) group is susceptible to hydrogen ion, water, and hydroxyl ion catalyzed hydrolysis with evidence for general base catalysis as by monohydrogen phosphate ion. The apparent first-order rate constant, *k*, for this hydrolysis may be defined as

<sup>2</sup> As originally suggested on organochemical evidence by Dr. Heinz Jahnke of these laboratories.

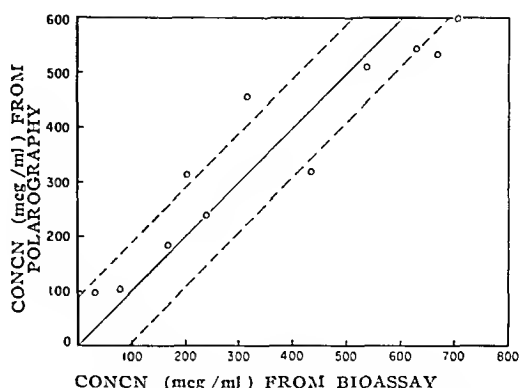


Fig. 12—Attempted correlation of streptozotocin polarographic assay with bioassay on material degraded at 30°, pH = 3.42 (run No. 26). The solid line is the theoretical relation of unit slope. The dashed lines represent the standard deviation of bioassay about regression.

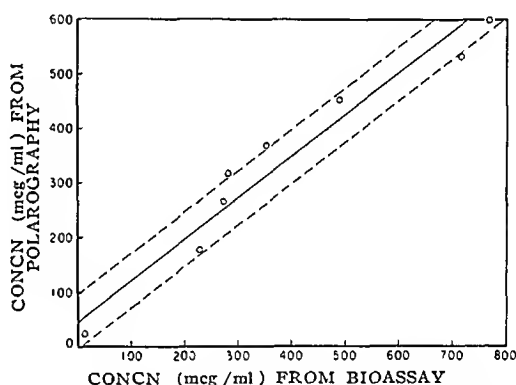


Fig. 13—Attempted correlation of streptozotocin polarographic assay with bioassay on material degraded at 30°, pH = 5.86 (run No. 31). The solid line is fitted by least squares, the dashed lines represent the standard deviation of bioassay about regression.

$$k = k_H + [\text{H}^+] + k_{\text{HPO}_4^-} [\text{HPO}_4^-] + \frac{k_{\text{OH}^-}}{[\text{OH}^-]} + k_0 \quad (\text{Eq. 25})$$

where the specified rate constants at various temperatures have been given previously. The *k<sub>H</sub>* + rate constant is actually a synthesis of two others, the one representing hydrogen ion attack on a protonated, the other on a nonprotonated streptozotocin of pKa = 1.35 at 30°, possibly due to the N-nitrosomethylamide group of weak basicity.

**Prediction of Stability in Vitro and in Vivo.**—The optimum pH for the maintenance of streptozotocin stability in solution is ca. pH 4.0 where the antibiotic has a half life of 90 hours at 30°. At a pH of 1.0, the half life is 14 hours, and at a pH of 7.0 it is 3.84 hours at 30°. It follows that significant degradation can be expected in the stomach on oral ingestion although the loss in potency prior to absorption would not be of great practical significance if a maximum of one to two hours at a pH of 1–1.5 for stomach retention is assumed.

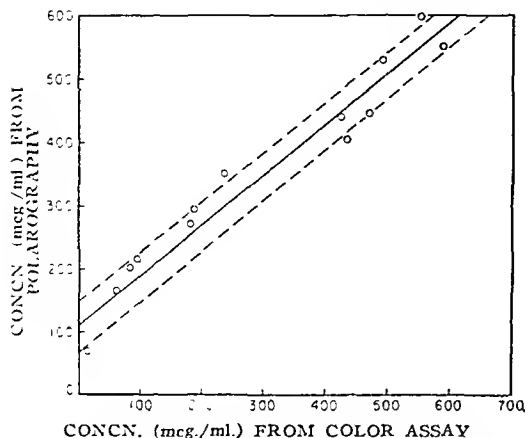


Fig. 14.—Attempted correlation of streptozotocin polarographic assay with bioassay on material degraded at 30°, pH = 2.02 (run No. 25). The solid line is fitted by least squares; the dashed lines represent the standard deviation of bioassay about regression.

However, the important factor to consider in *in vivo* stability is the possible degradation in the intestine and in the blood. Fortunately, streptozotocin is nonionic so that a high degree of gastric absorption may be surmised from a site of inconsequential degradation. At a pH of 7 at 37°, the half life of streptozotocin is *ca.* one hour. It thus follows that no matter the metabolic or excretory rate of organism given the drug, the half life of the drug in the blood would not exceed one hour and would most probably be less. When the factors of intestinal degradation (pH 5–7) and distribution to tissues are considered, it is to be expected that it would be extremely difficult to pick up sensible blood levels of streptozotocin on oral administration.

**Comparison of Assay Procedures.**—The color assay is an effective and valid procedure above the pH range (> 5.0) where nitrite is not a product of degradation. Since the color assay measures nitrite yield from acid degraded streptozotocin by the diazotization of sulfanilic acid, nitrite contaminant would be calculated as streptozotocin. Such a contaminant must be compensated.

The color appearance at room temperature after the addition of the assay reagents is indicative of nitrite presence. The linear portion of a plot of color absorbance *versus* time for the first fifteen minutes may be extrapolated to zero time, as in Fig. 3. The resultant intercept value should be subtracted from the total color development at 60° and the difference used in the estimation of the streptozotocin present.

The polarographic assay of the functional group is independent of the nitrite present as an impurity and may be used without compensatory techniques on acidic solutions of the antibiotic. Although the polarographic assay correlates well with the bioassay (by plate-disk against *P. vulgaris*) at the neutral and mildly acidic pH values, it gives an apparently higher assay reading on streptozotocin degraded in strong acid. This may be due to the fact that streptozotocin has an additional mode of

degradation in strong acid thus destroying potency. This mode would involve some function other than the N-nitrosomethylamide grouping.

Correlation of bioassay with the polarographic assay (using only those degradations at pH values where the regression line goes through the origin) permits estimation of the error in the bioassay, a standard deviation of 80 mcg./ml. for streptozotocin in the concentration range 0–700 mcg./ml., average 350; or the standard deviation, % of average, is 23%.

The shift of half wave potential,  $E_{1/2}$ , (Fig. 2) to less negative potentials with increasing acetate ion concentration in the polarography of streptozotocin is an interesting phenomenon. The reduction by electrons of a compound is abetted by the addition of negative ions. It has been suggested<sup>1</sup> that streptozotocin complexes with the buffer in the region of the microelectrode. The polarographic procedure is valid, however, since at a given acetate buffer concentration, the  $i_D/C$  and  $E_{1/2}$  values for streptozotocin showed no significant variation with the concentration,  $C$ , of the antibiotic.

## SUMMARY

1. The antibiotic streptozotocin readily degrades in aqueous solution as a function of hydrogen and hydroxyl ion concentrations with a maximum stability at pH 4. Water and monohydrogen phosphate ion catalyzed hydrolysis are indicated.

2. The  $\log k - \text{pH}$  profile can be fitted by the postulate of differential hydrogen ion catalyzed hydrolysis on an ionic equilibrium with protonated streptozotocin,  $\text{pK}_a$  1.35 at 30°.

3. Prediction of *in vivo* stability of the antibiotic streptozotocin indicates that although gastric degradation would be of little importance on oral administration, degradation in the intestine and blood would be. It is anticipated that great difficulty would be encountered in obtaining significant blood levels since the half life in the blood should not exceed one hour.

4. A color assay based on diazotization of sulfanilic acid by the nitrite from acid degraded streptozotocin is valid providing nitrite is not a contaminant. Procedures are given to compensate for such a contaminant.

5. A polarographic assay is developed which can assay for streptozotocin in the presence of nitrite and is applicable in acid degrading systems.

6. The error in bioassay has been estimated and correlations have been demonstrated with color and polarographic assays of streptozotocin. The nitrite contaminant would interfere with the uncorrected color assay correlation. Only in acid solutions is the polarographic assay not fully correlated with bioassay. This indicates that acidic degradation which destroys biological potency proceeds by two mechanisms, the de-

struction of the reducible N-nitrosomethylamide and some other functionality independent of the group. Thus when the biological potency is zero, some reducible N-nitroso may remain.

7. Potentiometric titrations, the results of the kinetic studies, and the correlations indicate that streptozotocin is an N-nitrosomethylurea and this function's stability, for the greater part, is correlated with the biological activity of the antibiotic. Mechanisms of solution degradation have been proposed.

## REFERENCES

- (1) Garrett, E. R., *THIS JOURNAL*, **48**, 169 (1959)
- (2) Vavra, J. J., DeBoer, C., Dietz, A., Hanka, L. J., and Sokolski, W., *Antibiotics Ann.*, **1959/60**, 230
- (3) Herr, R. R., Ehle, T. E., Bergy, M. E., and Jahnke, H. K., *ibid.*, **1959/60**, 236
- (4) Sokolski, W. T., Vavra, J. J., Hanka, L. J., and Burch, M. R., *ibid.*, **1959/60**, 241
- (5) Lewis, C., and Barbiers, A. R., *ibid.*, **1959/60**, 247
- (6) Hanka, L. J., Sokolski, W. T., and Vavra, J. J., *ibid.*, **1959/60**, 255
- (7) Forist, A. A., *Anal. Chem.*, in press
- (8) Edwards, L. J., *Trans. Faraday Soc.*, **46**, 723 (1950)
- (9a) Haroed, H. S., and Oweo, B. B., "The Physical Chemistry of Electrolytic solutions," 3rd edition, Reinhold Publishing Co., New York, N. Y., 1958, p. 716
- (9b) *ibid.* p. 638

# Absorption Spectra of Cardiac Glycosides and Aglycones in Sulfuric Acid\*

By B. T. BROWN and S. E. WRIGHT

The absorption spectra of 38 cardiac glycosides, aglycones, and aglycone derivatives, have been determined in sulfuric acid. The use of these spectra may assist in differentiating between members of the A, B, and C series of digitalis glycosides and aglycones.

THE USE of absorption spectra in concentrated sulfuric acid for the characterization and estimation of steroids has become an important aid in the study of these compounds. The initial work by Zaffaroni and co-workers (1, 2) has been supplemented by the studies of Bernstein and Lenhard (3, 4), and the absorption spectra of several hundred steroids in sulfuric acid are now recorded. Certain structural features of the steroid molecule have been related to selective absorption in sulfuric acid, including C-3, $\Delta^{4,5}$  unsaturated carbonyl group which has been correlated with absorption in the 280–300  $m\mu$  region, and isolated carbonyl and hydroxyl groups which absorb in the 239–249  $m\mu$  region (4).

While the absorption spectra in ethanol of many cardiac glycosides and aglycones have been recorded (5), the application of sulfuric acid spectra of these compounds for identification purposes has been limited. Murphiv (6) recorded the absorption spectra in sulfuric acid of four aglycones in the region 360 to 600  $m\mu$ . The

present paper is a study of the absorption spectra in sulfuric acid of 38 glycosides, aglycones, and aglycone derivatives in the range 210 to 600  $m\mu$ , and the spectra recorded have provided a useful method of helping to distinguish between digitalis glycosides and aglycones of the A, B, and C series.

## EXPERIMENTAL

**Determination of Absorption Spectra.**—Absorption spectra were determined on a Hilger "Uvispec" spectrophotometer model SP 500 (Hilger and Watts, Ltd.). Readings were recorded at 5- $m\mu$  intervals. Silica cells (1 cm) were used throughout, a hydrogen lamp in the range 210–360  $m\mu$ , and a tungsten lamp 360–600  $m\mu$ .

The solutions for analysis were in a concentration of 15 mg/ml to 30 mg/ml, and were determined within one hour after solution in sulfuric acid (98% w/w  $H_2SO_4$ ). To investigate the alteration of spectra with time, the spectrum of each compound was redetermined after twenty-four hours.

**Tabulation of Data.**—In Table I, the absorption maxima and minima  $E(1\%, 1\text{ cm})$  for each compound have been recorded. Each absorption band is recorded by a single wavelength and the symbol (I) has been used to denote an inflection or a plateau if this was observed in the spectra.

\* Received May 9, 1960 from the Department of Pharmacy, University of Sydney, Sydney, Australia.  
The authors would like to thank Mr. C. Short for assistance with the experimental work.



TABLE I—ABSORPTION SPECTRA OF COMPOUNDS STUDIED

No	Compound	Maxima $m\mu$ <sup>c</sup>	Minima $m\mu$ <sup>c</sup>
Cardiac Glycosides			
1	Lanatoside A <sup>a</sup>	235 (150), 325 (175), 415 (110), 480 (95) (I)	285 (90), 375 (90)
2	Digitoxin <sup>a</sup>	235 (270), 340 (240), 420 (240), 485 (230)	265 (175), 375 (215), 440 (165)
3	Desacetyl lanatoside B <sup>a</sup>	315 (180), 420 (140), 500 (310) (I), 530 (360)	255 (115), 360 (115), 445 (120)
4	Gitoxin <sup>b</sup>	235 (210) (I), 315 (275), 415 (185), 495 (430), 530 (505)	260 (150), 375 (140), 430 (160), 510 (405)
5	Digitalinum verum <sup>a</sup>	235 (85) (I), 320 (190), 495 (210) (I), 530 (280)	260 (150), 400 (30)
6	Digitalinum verum monoacetate <sup>a</sup>	235 (95) (I), 320 (230), 495 (240) (I), 530 (305)	240 (75), 390 (40)
7	Gitoside <sup>c</sup>	235 (140) (I), 310 (285), 500 (385) (I), 530 (475)	265 (105), 390 (100)
8	Lanatoside C <sup>a</sup>	230 (235), 320 (130) (I), 390 (295), 480 (160)	255 (105), 430 (105)
9	Acetyl digitoxin <sup>a</sup>	230 (195), 320 (105) (I), 390 (280), 490 (165)	260 (95), 360 (105), 440 (105)
10	Desacetyl lanatoside C <sup>a</sup>	235 (215), 325 (120), 390 (260), 480 (180)	265 (95), 340 (110), 440 (105)
11	Digitoxin <sup>b</sup>	230 (260), 320 (225), 390 (305), 490 (210)	265 (110), 340 (130), 430 (130)
12	20,22-Dihydrodigitoxin <sup>d</sup>	320 (195), 390 (380), 490 (215)	260 (100), 345 (130), 440 (140)
13	Diginatin <sup>c</sup>	230 (185), 310 (175), 390 (315), 480 (230), 560 (120)	220 (170), 255 (95), 335 (160), 460 (180), 540 (105)
14	Afroside acetate <sup>d</sup>	235 (520), 340 (170) (I), 360 (205)	270 (60)
15	Gomphoside acetate <sup>d</sup>	235 (295), 355 (250)	270 (40)
16	Ouabain <sup>b</sup>	240 (450), 325 (215), 515 (80)	215 (270), 295 (170), 365 (45)
17	Strophanthin-k <sup>d</sup>	240 (195), 325 (220), 420 (130)	270 (90), 370 (60)
18	Scillaren A <sup>a</sup>	255 (160), 327 (425), 420 (85) (I), 530 (95)	230 (125), 270 (145), 390 (40)
19	Scilliroside <sup>a</sup>	295 (260), 505 (315)	230 (110), 330 (70)
Cardiac Aglycones and Derivatives			
20	Digitoxigenin <sup>d</sup>	235 (320), 350 (290), 410 (270)	270 (115), 385 (200)
21	20,22-Dihydrodigitoxigenin <sup>d</sup>	355 (170), 420 (280)	260 (85), 385 (130)
22	Digitoxigenin-3-acetate <sup>d</sup>	235 (370), 345 (290), 415 (270)	260 (95), 385 (170)
23	Digitoxigenin-3-one <sup>d</sup>	230 (180), 350 (530), 420 (50)	265 (25), 390 (15)
24	$\beta$ ( $\Delta^{14(15)}$ ) Anhydrodigitoxigenin <sup>d</sup>	235 (405), 350 (275), 410 (220)	260 (135), 385 (160)
25	$\beta$ -( $\Delta^{14(15)}$ )-Anhydrodigitoxigenin acetate <sup>d</sup>	235 (430), 350 (300), 415 (185)	260 (120), 375 (130)
26	Gitoxigenin <sup>d</sup>	225 (230) (I), 315 (420), 415 (120), 510 (400) (I), 540 (440)	260 (140), 365 (90), 430 (105)
27	$\Delta^{14(16)}$ Dianhydrodigitoxigenin <sup>d</sup>	235 (370), 350 (240), 420 (270)	215 (250), 270 (105), 395 (175)
28	Digitoxigenin <sup>d</sup>	230 (410), 320 (310), 390 (350), 485 (110)	260 (95), 340 (100), 430 (90)
29	Digitoxigenin-3,12 diacetate <sup>d</sup>	230 (330), 320 (350), 390 (100), 490 (70)	260 (75), 340 (45), 410 (40)
30	20,22-Dihydrodigitoxigenin <sup>d</sup>	315 (150), 390 (450), 485 (160)	250 (95), 340 (105), 430 (85)
31	Digitoxigenin-3 one <sup>d</sup>	230 (415), 320 (410), 390 (140)	215 (390), 260 (130), 350 (85)
32	$\beta$ -( $\Delta^{14(15)}$ )-Anhydrodigitoxigenin <sup>d</sup>	230 (350), 320 (120), 390 (360), 520 (100)	260 (75), 345 (100), 450 (80)
33	Digitoxigenin 3,12 dione <sup>d</sup>	230 (380), 315 (230), 390 (260), 500 (80) (I)	260 (100), 350 (125), 430 (80)
34	Diginatigenin <sup>c</sup>	230 (160), 310 (130), 390 (210), 425 (190) (I), 490 (85)	250 (45), 345 (85), 470 (75)
35	Sarmentogenin <sup>a</sup>	230 (460), 415 (430)	215 (360), 270 (75)
36	11- $\alpha$ - $\beta$ -Sarmentogenin <sup>a</sup>	230 (230), 415 (410)	275 (60)
37	Afroside- $\beta$ anhydrogenin acetate <sup>d</sup>	230 (240), 340 (95) (I), 360 (120)	260 (45)
38	Orobagenin <sup>c</sup>	240 (510), 295 (190) (I), 420 (125) (I), 510 (210)	375 (75)

Sources of material: <sup>a</sup> Sandoz A. G. Basel; <sup>b</sup> Burroughs Wellcome & Co. (Aust.) Ltd. Sydney, Australia; <sup>c</sup> Burroughs Wellcome & Co. New York, N. Y.; <sup>d</sup> Department of Pharmacy, University of Sydney, Sydney, Australia.

<sup>c</sup> The figures in parentheses refer to the  $F(1\% \text{ 1 cm})$  value at the wavelength stated.

## DISCUSSION

In the present series of compounds, there is little difference between the absorption spectra of glycosides and of aglycones. Thus, the spectra of digoxin (No. 11) and gitoxin (No. 4) are very similar to that of their respective aglycones, digoxigenin (No. 28) and gitoxigenin (No. 26). Any differences are in intensity rather than in the positions of the maxima. It would appear, therefore, that the absorption maxima in sulfuric acid are due principally to the steroid aglycone and the groups substituted in this nucleus. Comparison of the spectra of digoxigenin (No. 28) and diacetyl digoxigenin (No. 29), and of digitoxigenin (No. 30) with digitoxigenin-3-one indicates that acetylation of hydroxyl groups or their oxidation to ketone has little effect upon the absorption spectra.

In ethanol, the  $\Delta^{20,22}$  unsaturated lactone of the C-17 cyclobutenolides absorbs at 217  $m\mu$  [range 215 to 218  $m\mu$  (6)], while compounds in which this double bond has been reduced do not show absorption in this region. In sulfuric acid, the unsaturated butenolides examined all showed absorption at 235  $m\mu$  (230 to 240  $m\mu$ ). The absorption spectra of each of the three 20,22-dihydro derivatives examined in the present study [20,22-dihydrodigoxin (No. 12), 20,22-dihydro-digitoxigenin (No. 21), and 20,22-dihydro-digoxigenin (No. 30)] do not show an absorption band at 235  $m\mu$ , but each spectrum does show other absorption maxima corresponding to those present in the corresponding unsaturated cyclobutenolide. It would appear that the absorption maximum at 235  $m\mu$  can be assigned to the cyclobutenolide ring, the maximum being shifted from 217  $m\mu$  in ethanol to 235  $m\mu$  in sulfuric acid.

The absorption maxima of the present series of compounds in sulfuric acid appear to be dependent on the position and nature of groups substituted in the aglycone nucleus. These spectra can therefore assist in differentiating between small amounts of closely related aglycones or aglycones derivatives. For example, while the  $R_f$  values of digoxigenin (a 3 $\beta$ , 12 $\beta$ , 14 $\beta$  trihydroxy compound) and sarmen-togenin (a 3 $\beta$ , 11 $\alpha$ , 14 $\beta$  trihydroxy compound) are similar in a number of paper chromatography systems, the sulfuric acid absorption spectra are quite different, and microgram amounts of these aglycones have been distinguished by this latter procedure (7, 8).

The absorption spectra in sulfuric acid of the digitalis glycosides provide a convenient method for distinguishing between members of the A (digitoxigenin derivatives), B (gitoxigenin derivatives), or C (digoxigenin derivatives) series of glycosides. It is evident that wavelengths of absorption maxima depend on the number and position of nuclear hydroxyl groups attached to the aglycone nucleus, and any sugar residue at C-3 only modifies peak intensity. Table II records the absorption maxima of digitalis glycosides and aglycones of the A, B, and C series.

The A series compounds (with 3 $\beta$ , 14 $\beta$  hydroxyl groups) may be distinguished by maxima at 350 and 415  $m\mu$ , the B series by strong maxima at 490 and 530  $m\mu$ , and the C series by maxima at 320 and 390  $m\mu$ .

TABLE II.—ABSORPTION MAXIMA OF DIGITALIS GLYCOSIDES AND AGLYCONES OF A, B, AND C SERIES IN SULFURIC ACID

Digitalis Series	Absorption Maxima, $m\mu$				
A series	235	350	415		
B series	235	320	415	490	530
C series	235	320	390		

It is interesting to note that the prominent maxima at 490 and 530  $m\mu$  which are characteristic of the B digitalis series, are absent in the spectrum of  $\Delta^{14,16}$ -dianhydrogitoxigenin (No. 27). The spectrum of this compound resembles rather that of the A series (3 $\beta$ , 14 $\beta$  hydroxyls) with maxima at 235, 350, and 420  $m\mu$ . It would appear then that the prominent B series maxima at 490 and 530  $m\mu$  are dependent on the presence of a C-16 hydroxyl group.

The absorption spectra recorded twenty-four hours following solution in sulfuric acid generally indicated only slight changes in maximal absorption values. However, the following differences were observed. After twenty-four hours, the spectra of afroside acetate (No. 14), afroside  $\beta$ -anhydrogenin acetate (No. 37), and gomphoside acetate (No. 15) had developed maxima at 300  $m\mu$ ,  $E(1\%, 1\text{ cm.})$  260, 130, and 250, respectively, and the previous maxima at 340 and 360  $m\mu$  had disappeared. After twenty-four hours digitoxigenin-3-one (No. 23) developed a maximum at 290  $E(1\%, 1\text{ cm.})$  215 and the maximum at 350  $m\mu$  had disappeared. The maximum at 390  $m\mu$  for both digoxigenin (No. 28) and digoxigenin diacetate (No. 29) increased considerably during twenty-four hours from initial  $E(1\%, 1\text{ cm.})$  values of 350 and 100, respectively, to 530 and 430, while the 320  $m\mu$  maximum of both compounds decreased to  $E(1\%, 1\text{ cm.})$  180 and 130, respectively.

## SUMMARY

The absorption spectra of 38 cardiac glycosides, aglycones, and aglycone derivatives in 98 per cent sulfuric acid have been determined from 210 to 600  $m\mu$ . The presence of an absorption peak at 235  $m\mu$  (range 230–240  $m\mu$ ) indicates the presence of a cyclobutenolide group. The use of sulfuric acid absorption spectra may assist in distinguishing between the A, B, and C series of digitalis glycosides and aglycones.

## REFERENCES

- (1) Zaffaroni, A., *J. Am. Chem. Soc.*, **72**, 3828(1950).
- (2) Diaz, G., Zaffaroni, A., Rosenkranz, G., and Djerassi, C., *J. Org. Chem.*, **17**, 747(1952).
- (3) Bernstein, S., and Lenhard, R. H., *ibid.*, **18**, 1146(1953).
- (4) Bernstein, S., and Lenhard, R. H., *ibid.*, **19**, 1269(1954).
- (5) Dorfman, L., *Chem. Revs.*, **53**, 47(1953).
- (6) Murphy, J. E., *THIS JOURNAL*, **44**, 719(1955).
- (7) Brown, B. T., Wright, S. E., and Okita, G. T., *Nature*, **180**, 607(1957).
- (8) Ashley, J. J., Brown, B. T., Okita, G. T., and Wright, S. E., *J. Biol. Chem.*, **232**, 315(1958).

# *In Vitro* Antibacterial Activity of Oils from Indian Medicinal Plants I\*

By C. L. CHOPRA, M. C. BHATIA, and I. C. CHOPRA

*In vitro* antibacterial activity of nine essential oils and one fixed oil obtained from Indian medicinal plants was determined using a filter paper disk method. The oils were tested against various Gram-positive and Gram-negative organisms. All the oils tested were found to be effective against one or more test organisms.

SOME VOLATILE principles from Indian medicinal plants have long been used for the treatment of infective diseases, both local and systemic. Some of the essential oils isolated in this institute and their antimicrobial activity by serial dilution method have been reported earlier (1-5). Maruzella, *et al.* (6-9), reinvestigated some known oils, singly and in combination, for their antibacterial activity by filter paper disk method. The present communication deals with antimicrobial activity of some further essential oils and one fixed oil from the following medicinal plants by filter paper disk method.

*Artemisia absinthium* Linn. (*Compositae*). Absinth.—The herb is an aromatic tonic and formerly enjoyed a high reputation in the treatment of debility and disorders of the digestive organs. It is prescribed in the form of a poultice as an antiseptic and discutient.

*Cedrus deodara* (Roxb.) Loud syn. *C. libani* Barrel var. *Deodara* Hook f. (*Pinaceae*), Himalayan cedarwood.—In ancient systems of medicine all parts of the plant were considered useful in belching, inflammations, fever, urinary discharges, bronchitis, itching, tuberculous glands, ophthalmia, piles, etc. The oil was considered useful in bruises and injuries to joints, boils, tubercular glands, and skin diseases.

*Ferula jaeschkeana* Vatke (*Umbelliferae*).—The gum-resin is applied to wounds and bruises by the inhabitants of Kurram valley.

*Origanum vulgare* Linn. (*Labiatae*) Marjoram.—The plant was considered useful in inflammations, catarrh, ear infections, bronchitis, and asthma.

*Pinus excelsa* Wall. syn. *P. griffithii* M'Clell (*Pinaceae*).—The plant was considered useful in ophthalmia, liver troubles, chronic bronchitis, and griping. The seed oil is highly esteemed for its stimulating and healing powers when applied as a dressing to wounds and ulcers.

*Pinus longifolia* Roxb. syn. *P. roxburghii* Sargent (*Pinaceae*), Long Leaved Pine.—The plant was considered useful in diseases of the eye, ear, throat, skin, bronchitis, ulcers, inflammations and itching, asthma, urinary discharges, and tuberculous glands.

*Punica granatum* Linn. (*Punicaceae*) Pomegranate.—The Hindu and Greek systems of medicine consider the herb and seeds to be useful in bronchitis, sore throat, and stomatitis. The rind of the fruit is employed as anthelmintic and in diarrhea and dysentery. The fresh juice of the fruit is used as an ingredient of some mixtures considered useful in dyspepsia.

*Saussurea lappa* Clarke (*Compositae*) Costus.—The root is bitter, sweetish, and pungent, and enjoys a reputation as aphrodisiac and alterative and as a cure for leucoderma, erysipelas, itching, ringworm, bronchitis, and scabies. According to Greek medicine the root cures diseases of the liver and kidneys, pain in the chest, asthma, cough, inflammations, ophthalmia, and old fevers. The Chinese considered the root to be carminative and stimulant.

*Zanthoxylum alatum* Roxb. (*Rutaceae*).—The fruit is reputed to cure pain, tumors, abdominal troubles; is considered useful in eye and ear diseases, leucoderma, asthma, and troubles of the spleen.

## EXPERIMENTAL

The detection of the antibacterial activity of various oils was made by observing their effects on the cultures of the following ten organisms: *Escherichia coli*, *Staphylococcus aureus*, *Acrobacter aerogenes*, *Salmonella typhosa*, *Salmonella paratyphi*, *Salmonella typhosa* vi, *Shigella flexneri*, *Proteus vulgaris*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*.

Vincent's method as described by Maruzella, *et al.* (6), was used for determining the presence or absence of antimicrobial activity. Eighteen-hour cultures were used for seeding 2% nutrient agar used for the tests. Sterile disks of 12.5-mm. diameter were saturated with the oil and placed on the seeded agar. The presence of definite zones of inhibition of any size indicated positive antimicrobial activity. For measuring the size of the zones, the filter paper disks were removed and diameter of the zones showing no growth after twenty-four hours of incubation were measured to the nearest millimeter by means of a divider, the plates being suitably illuminated in Quebec colony counter. All tests were conducted in

\* Received November 23, 1959, from the Regional Research Laboratory, Jammu (Kashmir), India.

The authors acknowledge the technical assistance of S. N. Kak.

TABLE I—ANTIBACTERIAL ACTIVITY OF OILS FROM MEDICINAL PLANTS\*

Plants	Part from Which Oil Was Extracted	Zones of Inhibition, mm									
		<i>E. coli</i>	<i>Staph aureus</i>	<i>Aerobacter aerogenes</i>	<i>S. typhosa</i>	<i>S. paratyphi</i>	<i>S. typhosa vi</i>	<i>Sh. flexneri</i>	<i>Pr. vulgaris</i>	<i>Kl. pneumoniae</i>	<i>Ps. aeruginosa</i>
<i>A. absinthium</i>	Whole plant			13					13	13	14
<i>C. deodara</i>	Leaves	28		13			15	28	13	14	13
<i>C. deodara</i>	Wood	15		13					13	13	13
<i>F. jaeschkeana</i>	Root			13					13	13	14
<i>O. vulgare</i>	Whole plant			17					14	16	14
<i>P. longifolia</i>	Leaves	20	17	14		18	13	14	13	13	14
<i>P. excelsa</i>	Leaves	.	25	13		24		40	21	13	13
<i>P. granatum</i> (fixed oil)	Seeds		..		15	16		14		13	
<i>S. lappa</i>	Root		17	14				25	13	14	14
<i>Z. alatum</i>	Whole plant	13		17		14			15	16	13

\* Measurements recorded are the averages of six separate tests

triplicate, with two disks per plate, each reading being an average of six observations. In no instance was complete clearing of the plates observed. See Table I.

The essential oils used in the investigations were prepared by steam distillation of the dried plant material. The oils were separated from the aqueous layer and the remaining portions of the distillate saturated with sodium chloride. They were then extracted with ether and the ether distilled off. The oils so obtained were mixed with the oils already obtained and dehydrated.

Fixed oil was obtained by extracting the powdered seeds with petroleum ether in a Soxhlet extractor. The extract was decolorized by animal charcoal. The petroleum ether was distilled off and the oil so obtained was used for antibacterial investigations.

The oils were immediately placed in sterile test tubes and tested for sterility. Each oil was streaked on nutrient agar and Sabouraud's dextrose agar slants and also inoculated in nutrient-broth tubes. The tubes were incubated at 37° for five days. All the oils were found to be sterile.

## RESULTS AND DISCUSSION

The table indicates that each of the ten oils tested possessed some antimicrobial activity on one or more of the organisms. The essential oil from *Pinus excelsa* was found to be the most potent of the oils, giving wider zones of inhibition, though a comparatively lesser species of bacteria were sensitive to it. The essential oil of *P. longifolia* affected the growth of maximum number of organisms and was the next in order of potency. The oil from the leaves of *Cedrus deodara* also inhibited seven organisms, exhibiting quite large zones of inhibition in case of *E. coli* and *Sh. flexneri*. All other oils are relatively less potent.

Investigations conducted out by Maruzzella and Lichtenstein (6) show that, generally, fixed oils exhibit no antibacterial activity whatsoever whereas present studies show the fixed oil obtained from the seeds of *P. granatum* possess some antibacterial

activity against *S. typhosa*, *S. paratyphi*, *Sh. flexneri*, and *Kl. pneumoniae*. This oil is not as potent as some of the other essential oils but its activity appears to be better than the essential oils of *A. absinthium* and *F. jaeschkeana*.

No correlation could definitely be established between the antibacterial activity of the essential oils and their reputed use in the ancient systems of medicine. For example, in the medicinal uses of *S. lappa* no particular infectious disease has been mentioned but it still exhibits antibacterial activity against *S. aureus*, *Aerobacter aerogenes*, *Sh. flexneri*, *Pr. vulgaris*, *Kl. pneumoniae* and *Ps. aeruginosa*.

## SUMMARY

1 Nine essential oils and one fixed oil from the Indian medicinal plants alleged to have some therapeutic value were tested for their *in vitro* antibacterial activity against ten common organisms using the filter paper disk technique.

2 The essential oil obtained from the leaves of *P. excelsa* was found to be the most potent one. Next in order of potency are *P. longifolia*, *C. deodara*, and *S. lappa*. The remainder of the essential oils were comparatively less potent.

3 Fixed oil from the seeds of *P. granatum* exhibited inhibitory zones against *S. typhosa*, *S. paratyphi*, *Sh. flexneri* and *Kl. pneumoniae*.

## REFERENCES

- (1) Chopra, I. C., Gupta, K. C., and Nazir, B. N., *Indian J. Med. Research*, 40, 511 (1952).
- (2) Gupta, K. C., and Chopra, I. C., *ibid.*, 41, 427 (1953).
- (3) Gupta, K. C., and Chopra, I. C., *ibid.*, 42, 355 (1954).
- (4) Chopra, I. C., Khajuria, B. N., and Chopra, C. L., *Antibiotics & Chemotherapy*, 7, 378 (1957).
- (5) Chopra, I. C., and Chopra, C. L., *Indian J. Med. Research*, 47, 161 (1959).
- (6) Maruzzella, J. C., and Lichtenstein, M. B., *This Journal*, 45, 378 (1956).
- (7) Maruzzella, J. C., and Liguari, L., *ibid.*, 47, 250 (1958).
- (8) Maruzzella, J. C., and Henry, P. A., *ibid.*, 47, 294 (1958).
- (9) Maruzzella, J. C., and Henry, P. A., *ibid.*, 47, 471 (1958).

# Metabolic and Morphological Changes Induced by Gibberellic Acid on Spearmint\*

By EDWARD E. GONZALEZ† and GUNNAR GJERSTAD

The influence of gibberellic acid on the growth of spearmint and on the production of the volatile oil has been studied. Spearmint plants treated once throughout their growth period with foliar sprays of gibberellic acid in a concentration of 50 p. p. m. displayed a 258 per cent increase in linear growth. The experimental plants developed a very thick, wood-like stem which had an extended xylem region and missing ribs. An increase in dry matter and a decrease in water content of the tissues were found. Gibberellic acid treatment decreased percentage-wise the amount of volatile oil but did not alter its carvone content. The oil met all N. F. standards. A decrease in total chlorophyll content was found.

SINCE the discovery by Kogl in 1934 of the plant growth hormones hardly any chemical has attracted the attention of so many plant researchers as have the gibberellins. Although the effects of this group of substances which are the metabolic products of the parasitic ascomycete, *Gibberella fujikuroi*, were described by Konishi as early as 1828 (1), it was not till 1938 that a group of Japanese plant physiologists succeeded in isolating the main principle in a pure form. Up to January of 1959 some 800 publications on this subject had appeared. Reference is made to excellent review articles by Stodola (1) and Stowe and Yamaki (2).

Reviewing the literature up to 1957, when our research started, we failed to find any publication relating to the study of the possible effect of gibberellic acid on drug plants. We were interested in testing whether or not gibberellic acid would affect drug quality by altering the amount of active ingredients present. We chose spearmint for our study because apparently no volatile oil containing plants had been studied in this regard and because preliminary screening of various species and genera of drug plants in our drug garden indicated that *Mentha spicata* L. showed the greatest response to gibberellic acid.

Specific objectives of the present study were (a) to ascertain whether gibberellic acid would bring about any change in the amount of volatile oil in spearmint, (b) to establish if the percentage of carvone in the oil would be altered, and (c) to determine if any macro- and/or micromorphological changes are effected by gibberellic acid.

## EXPERIMENTAL

**Materials and Methods.**—Preliminary tests with gibberellic acid were started in the fall of 1957 and continued through the spring of 1958. In order to

ascertain that the results were reproducible, the main experiments were repeated during the 1958-1959 growing season.

Spearmint plants used in this study were grown in pot cultures under carefully controlled greenhouse conditions. The plants were propagated from stolons obtained from authenticated spearmint plants cultivated for several years in the University of Texas drug garden.<sup>1</sup> Three-inch-long stolons were planted about two inches deep in 6-inch clay pots containing soil composed of three parts of sandy loam and one part of sheep manure, because soil analyses indicated that organic fertilization was necessary. Forty-one days after planting, the young spearmint plants had attained a height of approximately two inches, and were transplanted to bigger pots containing the same type of enriched soil. After the plants had reached a height of about five inches, 60 pots were selected for study and divided randomly into two equal groups. One group was kept as a control, while the other was treated with a freshly prepared aqueous solution of gibberellic acid<sup>2</sup> (50 p. p. m.) prepared with a few drops of ethanol as solubilizer. The solution was administered in the form of a mist to the leaves and tops by use of a conventional spray gun. Each of the treated plants received approximately 65 ml of solution. After 120 days another spray of identical strength was applied.

In order to make sure that the plants received the proper nourishment for maturation and also in an attempt to avoid the chlorotic condition, observed in preliminary experiments, the soil was saturated every third week with nutrient solutions of the following compositions:

### Modified Hoagland's Solution

Ca(NO <sub>3</sub> ) <sub>2</sub> ·4H <sub>2</sub> O . . . . .	9.44 Gm.
KNO <sub>3</sub> . . . . .	4.00 Gm.
MgSO <sub>4</sub> ·7H <sub>2</sub> O . . . . .	9.80 Gm.
NH <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> . . . . .	4.60 Gm.
Distilled H <sub>2</sub> O to make . . . . .	8,000 cc.

### Solution of Trace Elements

CuCl <sub>2</sub> ·2H <sub>2</sub> O . . . . .	0.05 Gm.
H <sub>3</sub> BO <sub>3</sub> . . . . .	2.66 Gm.
MnCl <sub>2</sub> ·4H <sub>2</sub> O . . . . .	1.81 Gm.
ZnCl <sub>2</sub> ·2H <sub>2</sub> O . . . . .	0.11 Gm.
Distilled H <sub>2</sub> O to make . . . . .	1,000 cc.

\* Received November 7, 1959, from the College of Pharmacy, The University of Texas, Austin 12.

† Recipient of Kilmer Prize for 1959.

Presented to the Scientific Section, A. P. H. A., Cincinnati meeting, August 1959.

<sup>1</sup> Kindly furnished by Dr. C. C. Albers, University of Texas, Austin.

<sup>2</sup> Kindly supplied by Dr. Curt Leben of Eli Lilly Co., Indianapolis, Ind.

**Growth Effects.**—The spearmint plants were observed every week for specific growth effects. At various intervals throughout the growth period the height of each plant was measured from the base of the stem to the growing tip of the plant (Table I). In the preliminary experiments as well as in the second growing season, a demonstrable difference between the treated and the untreated plants could be noted twelve days after treatment

TABLE I.—INCREASES IN HEIGHT OF GIBBERELLIN-TREATED SPEARMINT PLANTS

Date Measured	Growth, %
December 10, 1958	. <sup>a</sup>
December 19	41
January 7, 1959	159
January 14	185
February 11	258
March 4	174
April 9	126 <sup>a</sup>
May 6	157
June 3	137

<sup>a</sup> Treated with 50 p p m

The most pronounced physiological response was a remarkable increase in height due mainly to an increase in length between the internodes of the stem (Fig. 1) Compared with the controls, the treated plants displayed increases in height of about 41% after two weeks and approximately 258% after nine weeks (Table I). Most of the treated plants de-

veloped initially very thin, thread-like stems which exhibited vine-like characteristics Most of these elongated stems thickened as the plants matured, however, into reddish purple main stems which were very hard and rough, longitudinally wrinkled, and irregular in shape (Fig 2) The main stems of the treated plants showed from four to six times greater diameter than those of the controls Also, an increase in lateral branching from the main stem was observed

It was noted that the individual leaves of the treated plants had less surface area and less succulent growth than those of the controls. Unlike the lamina of the controls which were ovate in shape, those of the treated plants were almost oblanceolate, rougher, longer, thinner, and appeared slightly chlorotic. In general, the treated plants did not appear to be as normal or healthy looking as the controls There was no evidence that gibberellic acid could induce spearmint plants to initiate flower primordia

Another noteworthy response was that the treated plants appeared to be more susceptible to insect attacks A similar observation has also been reported by Smith and Seiechetti (3) Our plants were repeatedly attacked by microscopic spider mites of the *Tetranychidae* family, which could be controlled with aqueous solutions of Destruxol, a commercial insecticide containing nicotine 0.6%, ammonia 0.8%, pine oil 5.5%, emulsified in mineral oil



Fig. 1.—Growth effects on *Mentha spicata* twenty-five weeks after the first treatment with gibberellic acid (50 p. p. m.). Left, first three plants were treated twice. Right, control. From left to right the plants measured 132, 120, 124, 38, 41, and 34 cm.



Fig 2.—Effect of gibberellic acid on the stems of *Mentha spicata*. Left, stem from an untreated plant; right, stem from a plant treated twice with 50 p. p m gibberellic acid. Both stems are approximately six months old. The ratio of stem thickness is 16:3 in favor of the treated plants. (Two-thirds natural size.)



Fig 3—Transverse sections of the stems of *Mentha spicata*. Top photograph, control; Bottom photograph, treated (50 p p m.). Note that the parenchyma cells in pith of the sections are larger in the control, but more numerous in the treated. Photomicrograph,  $\times 27$ .

**Influence on Histology.**—The morphology of the stem differed in that the xylem and cambium zones of the untreated stems are rectangularly shaped while those of the treated are more circular (Fig 3). As will be noted, the pith cells are larger in the controls but more numerous in the treated ones, the xylem tissue of the controls covers one-third of the area of the treated, and the ribs are missing in the experimental plants. The ratio of stem thickness is 16:3 in favor of the treated plants.

**Harvesting.**—It has been shown in the case of peppermint, that the volatile oil concentration reaches its peak at the start of flowering (4). Under the assumption that the same holds true for spearmint, harvesting was done when most of the plants were beginning to bloom. The plants were harvested in the laboratory in order to prevent unavoidable loss of water and volatile oil during transport. The plants were cut at soil level, rapidly weighed individually, and placed in extraction vessels.

### METHODS OF ANALYSIS

**Water and Oil Determinations.**—The plants were analyzed for water and volatile oil content according to the official methods in U. S. P. XV (5), and N. F. X (6), respectively. The results are presented in Table II.

**Carvone Analysis.**—The analysis of the carvone content in the spearmint oil was determined by ultraviolet absorption using a Beckman spectrophotometer model DU, in substance according to the procedure of Reitsema and Faas (7). All carvone determinations were performed at 320 m $\mu$  using a 0.323-mm slit width and 1-cm silica cells.

Before the oil was subjected to carvone analysis, it was carefully dried with anhydrous sodium sulfate. A sample of 0.10 ml of spearmint oil was then measured with a Kahn's blood capillary pipet, and diluted with 15 ml of methanol A. R. The prepared solution was then analyzed spectrophotometrically, and the corresponding per cent of carvone (Table II) was read from a prepared standard curve (Fig 4).

**Refractive Index.**—Since the refractive index is one of the best physical identity tests for volatile oils, this constant of the spearmint oil was determined according to the official method (6). The control oil showed a  $[n]_D^{20}$  of 1.4897, whereas that of the treated plants was established at 1.4906. Both values fall within the official range (6).

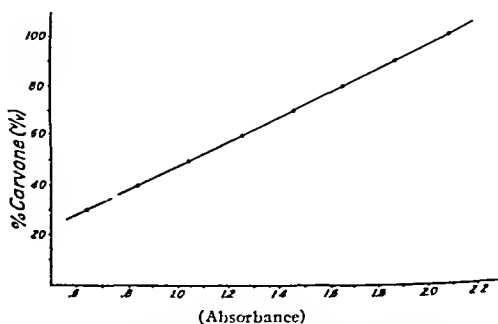


Fig 4—Calibration curve of l-carvone in methanol measured at 320 m $\mu$ .

TABLE II.—ANALYSIS OF WATER, VOLATILE OIL, AND CARVONE CONTENTS OF SPEARMINT PLANTS

Date of Harvest	Water, %		Volatile Oil, %		Carvone, %	
	C <sup>a</sup>	T <sup>a</sup>	C	T	C	T
June 3	82.25	61 15	0 613	0 433	56.10	57 50

<sup>a</sup> C, control, T, treated

**Chlorophyll Analysis.**—No quantitative analysis of the total chlorophyll content was performed since pure chlorophyll was not available. However, the total chlorophyll *a* and *b* contents of the leaves of treated and control plants were extracted according to a modified A. O. A. C. method (8), and the absorption of the solutions was measured in a Beckman spectrophotometer, model DU, using ultraviolet light source at a wavelength of 472  $\mu$ . The transmittance value of the control solution was 44.8% higher than in the treated plants, indicating that the chlorophyll concentration in the experimental plants is decreased.

### SUMMARY AND CONCLUSIONS

Preliminary experiments indicated that the applied concentration of gibberellic acid was not critical to ascertain demonstrable effects, inasmuch as no significant difference of response was observed after use of 10, 50, or 100 p. p. m.

The most conspicuous morphological response was the increased height of the treated plants, which became so weak that it was necessary to support them in order to keep them upright.

Spearmint plants treated with a solution of gibberellic acid (50 p. p. m.) showed the following responses:

1. A maximal increase in linear growth of 258 per cent.

2. A thick, red, round stem was developed by most of the treated plants.

3. The leaves were morphologically affected, developing less surface area and an oblanceolate shape.

4. Histologically, the pith cells were smaller and more numerous, the xylem area was larger and the ribs were missing.

5. A significant decrease in water content and a corresponding increase in dry weight were established (21.1 per cent).

6. A decrease in volatile oil production was found, but there were no significant changes in oil composition.

7. Gibberellic acid decreases the concentration of total chlorophyll.

### REFERENCES

- (1) Stodola, F. H., "Source Book on Gibberellin, 1828-1957," Agricultural Research Service, U. S. Department of Agriculture, Peoria, Ill., 1958.
- (2) Stowe, B. B., and Yamaki, T., *Science*, 129, 807 (1959).
- (3) Smith, G. M., and Seiuchetti, L. A., *THIS JOURNAL*, 48, 63 (1959).
- (4) Madaus, G., and Schindler, A., *Arch. Pharm.*, 276, 280 (1938).
- (5) "United States Pharmacopeia," 15th rev., Mack Publishing Co., Easton, Pa., 1955, p. 944.
- (6) "The National Formulary," 10th Ed., J. B. Lippincott Co., Philadelphia, Pa., 1955, p. 734.
- (7) Reitsema, R. H., and Faas, W. E., *THIS JOURNAL*, 46, 381 (1957).
- (8) "Official Methods of Analysis of the Association of Official Agricultural Chemists," 6th ed., A. O. A. C., Washington 4, D. C., 1945, p. 141.



## Notes

### Note on the Synthesis of Dihydroresorcinol\*

BY BERNHARD ESCH and HOWARD J. SCHAEFFER

THE PREPARATION of dihydroresorcinol has previously been accomplished by a variety of procedures. A high pressure hydrogenation at 50° of resorcinol has been described (1) in which yields of 85–95% may be obtained. A convenient synthesis for the preparation of dihydroresorcinol in an 85% yield by the low pressure, catalytic hydrogenation at room temperature of resorcinol using a 5% rhodium-on-alumina catalyst in the presence of sodium hydroxide has been developed. It has also been observed that the hydrogenation could be performed with a palladium-on-charcoal catalyst, but in this case no more than 82% of the theoretical amount of hydrogen was absorbed and the yields of the isolated product were in the 50–60% range.

#### EXPERIMENTAL

**Using a 5% Rhodium-on-Alumina Catalyst.**—To a solution of 1.46 Gm. of sodium hydroxide in 100 ml. of water was added 3.22 Gm. (29.2 mM) of resorcinol followed by 345 mg. of a 5% rhodium-on-alumina catalyst.<sup>1</sup> The mixture was hydrogenated overnight at an initial pressure of 60 psi and at room temperature, during which time the theoretical amount of the hydrogen was absorbed. The catalyst was removed by filtration, and the cooled filtrate was made acidic (pH 1–2) with concentrated hydrochloric acid, saturated with sodium chloride, and extracted with ethyl acetate (5 × 50 ml.). The combined organic extracts were dried with anhydrous magnesium sulfate, filtered, concen-

trated *in vacuo*, and gave 3.08 Gm. (94.3%) of crude dihydroresorcinol, m. p. 98–102°. The product was purified by sublimation at 0.5 mm. from an oil bath at 90°; yield, 2.82 Gm. (85.2%); m. p. 103–105°. The melting point was not depressed when the product was mixed with an authentic sample of dihydroresorcinol and the infrared spectrum of the product was identical with that of an authentic sample of dihydroresorcinol.

**Using a 5% Palladium-on-Charcoal Catalyst.**—To a solution of 2.52 Gm. of 85% potassium hydroxide in 100 ml. of water was added 3.30 Gm. (30 mM) of resorcinol followed by 307 mg. of a 5% palladium-on-charcoal catalyst.<sup>1</sup> The mixture was hydrogenated at an initial pressure of 30 psi. and at room temperature, but even after twenty-four hours no more than 82% of the theoretical amount of the hydrogen was absorbed. The catalyst was removed by filtration; the filtrate was taken to pH 5 with hydrochloric acid and then excess sodium bicarbonate was added. The solution was extracted with ethyl acetate (3 × 50 ml.) to remove the unchanged resorcinol; the aqueous layer was then made strongly acidic with concentrated hydrochloric acid and extracted with ethyl acetate (3 × 50 ml.). The combined organic extracts were dried with anhydrous magnesium sulfate, filtered, evaporated *in vacuo*, and gave 1.99 Gm. (59.4%) of the desired product, m. p. 103–108°. If desired, purification may be accomplished by sublimation as previously described.

#### REFERENCE

- (1) Thompson, R. B., "Organic Syntheses," Collective Vol. 3, John Wiley & Sons, New York, N. Y., 1955, p. 278

\* Received August 22, 1960, from the Department of Medicinal Chemistry, School of Pharmacy, The University of Buffalo, Buffalo, N. Y.

<sup>1</sup> Available from Engelhard Industries, Inc., Newark, N. J.

---

## Book Notices

---

*Pharmakologie* By VON KNUD O MØLLER  
Benno Schwabe & Co., Verlag, Basel, Stuttgart,  
1958 Available in the U S from Interconti-  
nental Medical Book Corp., 381 Fourth Ave.,  
New York 16, N Y 902 pp 17 × 24.5 cm

A comprehensive textbook and reference volume (in German) on pharmacology. A bibliography of 67 pages, a separate index referring to definitions of terms in the text, and a general subject index are appended.

*General Endocrinology* 3rd ed By C DONNELL  
TURNER W B Saunders Co., West Washing-  
ton Square, Philadelphia 5, Pa., 1960 vi +  
511 pp 15.5 × 23.5 cm Price \$9.50

Endocrinology is presented by the author from the experimental rather than the applied viewpoint. Almost all the clinical references in the earlier editions have been deleted and more comparative material added in this edition. A short section on the structure and nomenclature of steroid hormones now introduces the chapter on the adrenal cortex. The mechanisms of hormone action have been discussed as fully as present information permitted.

*Chemicals, Drugs and Health* By JOHN H FOUL-  
GER Charles C Thomas, 301-327 East Lawrence  
Ave., Springfield, Ill., 1960 viii + 102 pp 13.5  
× 21.5 cm

Some important situations faced by a "foreign" chemical when it tries to enter or has entered a living body are reviewed by the author. This was done to counteract apprehension that even very small amounts of new chemicals may ultimately affect health due to daily skin contact or inhalation.

*Non Benzenoid Aromatic Compounds* Edited by  
DAVID GINSBURG Interscience Publishers, Inc.,  
250 Fifth Ave., New York 1, N Y, 1959 vii +  
543 pp 15 × 23 cm Price \$18

Subjects covered by different authors are Aromaticity, Cyclobutadiene and related compounds, Compounds derived from cyclopentadiene, Pentalene and heptalene, Azulenes, Pathways to azulenes, Tropones and tropolones, Cyclooctatetraene, and Cyclopolyolefins. Author and subject indexes are appended.

*General Cytology* 3rd ed By E D P DeRo-  
BERTIS, W W NOWINSKI, and FRANCISCO A  
SAEZ W B Saunders Co., West Washington  
Square, Philadelphia 5, Pa., 1960 xvi + 555  
pp 15.5 × 23.5 cm Price \$10

Morphologic, physiologic, and genetic aspects of modern cytology are stressed in this revision of the book. A chapter on plant cells is one of five new chapters. Cytotoxic techniques for cell study are correlated with the results obtained and with considerations of progress in this field.

*Detoxication Mechanisms* 2nd ed By R TEEWYN  
WILLIAMS John Wiley & Sons, Inc., 440  
Fourth Ave., New York 16, N Y, 1960 xi +  
796 pp 14 × 21 cm Price \$19

The author feels that the subtitle "The Metabolism and Detoxication of Drugs, Toxic Substances and Other Organic Compounds" is a truer indication of the content of this book. The shorter title is retained because it has become well known in the twelve years since the first edition appeared. The manuscript for this edition was completed in 1957.

*Subsidia Pharmaceutica I, 1960 Supplement and Index of Names* Compiled and edited by the  
Scientific Center of the Swiss Pharmaceutical  
Society, and published by the Society, Zurich,  
1960 514 pp 17 × 24 cm Ring binder,  
loose-leaf

*Subsidia Pharmaceutica I*, a loose leaf compilation of pharmaceutical information, was first published in 1957 [for a review of the original work see THIS JOURNAL, 47, 763(1958)]. Supplements, issued periodically, extend the coverage of the work, and keep it up-to-date.

The 1960 Supplement (in German) comprises 42 pages and includes an excellent article on antihistaminic drugs in which the pharmacology and clinical uses are described, and a listing of names, structural formulas, and dosage forms of 43 such drugs is given. Other Supplement articles describe a modification of a prescription laboratory pressure-filtration apparatus suitable for filtering eye waters, a mobile drying and sterilizing cabinet, and two forms of disposable polystyrene and polyethylene molds for extemporaneously preparing and dispensing suppositories in the same unit.

The 1960 Index of Names is a 466 page alphabetic, cross referenced listing of international non-proprietary names, names used by various pharmacopoeias, chemical names, and brand names of drugs. The structural formula and principal pharmacologic category are provided for each drug.

Dr K Steiger-Trippi, Director of the Scientific Center of the Swiss Pharmaceutical Society, and his editorial associates have achieved commendably their objective of providing a variety of up-to-date information useful not only to pharmacists in Switzerland but also to their professional colleagues throughout the world.

*Advanced Organic Chemistry* 3rd ed By G W  
WHELAND John Wiley & Sons, Inc., 440 Fourth  
Ave., New York 16, N Y, 1960 xi + 871 pp  
15 × 23 cm Price \$17.50

The structural theory of organic chemistry is stressed and topics of special theoretical significance are included in the text. This edition includes electronic and nuclear magnetic resonance, conformational analysis, inclusion and charge transfer compounds, and the Hammett rho sigma relations.

*The National Formulary*. 11th ed. Prepared by the Committee on National Formulary under the supervision of the Council, by authority of the American Pharmaceutical Association. Published by the American Pharmaceutical Association, 1960. Distributed by J. B. Lippincott Co., East Washington Square, Philadelphia 5, Pa. xxxii + 531 pp. 15 × 23 cm. Price U. S. and Foreign, \$9.

The new National Formulary XI, which became official October 1, 1960, represents the results of an ambitious revision program. Extension of the scope of N. F. admissions to new drugs that have achieved wide use by the medical profession, but which were not admitted into the companion edition of the U. S. P., reflects the advance in practical value of this official compendium. Among the 148 completely new items in N. F. XI are Acetaminophen. (N-acetyl-*p*-aminophenol) (Category—Analgesic and antipyretic); Amisometradine (Category—Diuretic); Antazoline Phosphate (Category—Antihistaminic); Azacyclonol (Category—Psychotherapeutic agent); Benzestrol (Category—Estrogen); Benzpyrinium Bromide (Category—Parasympathomimetic); Benztropine Methanesulfonate (Category—Parasympatholytic); Dextromethorphan Hydrobromide (Category—Antitussive); Diphepanil Methylsulfate (Category—Anticholinergic); and Diphenadione (Category—Anticoagulant). These items, selected from those listed alphabetically under the letters A, B, C, and D, indicate the wide therapeutic spectrum that is covered. In reverse alphabetical order this interesting story reads the same. Zinc has finally yielded last place in the monograph section to Zoxazolamine (Category—Skeletal muscle relaxant; uricosuric). There are also Triacetyloleandomycin (Category—Antibiotic) and Rescinamine (Category—Tranquilizer; hypotensive). It is clearly evident that N. F. XI reflects the accelerated turnover in materia medica.

N. F. XI is bigger and better. It is Bigger—It contains 815 monographs (N. F. X had 733); it has 285 new items (137 from U. S. P. XV). It is Better—Its analytical procedures have been modernized and include column and paper chromatography, ultraviolet and infrared spectrophotometry, radioisotope tracer analysis, and countercurrent extraction. Its new two-column format makes reading easier; the page-top guide facilitates its use. The General Information section includes a really useful discussion of ophthalmic solutions with procedural guides for extemporaneous preparation. An important increase is noted in the 42 N. F. Reference Standards (N. F. X had 10). Most of the new reference standards are required in spectrophotometric analytical procedures. N. F. XI is smaller only in the number of pages. More material than ever has been included in 563 pages (N. F. X had 910 pages). This minor miracle was accomplished with the aid of experts at the Mack Printing Company. The two-column format, new type face, and style of arrangement is responsible for the elimination of much wasted space.

The value of the N. F. to pharmacists and others in the health professions is stepped up considerably in this latest edition.

*British Veterinary Codex Supplement 1959*. Published by direction of the Council of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, 1959. Obtainable in the U. S. from Rittenhouse Book Store, 1706 Rittenhouse Square, Philadelphia 3, Pa. xviii + 134 pp. 14.5 × 22.5 cm. Price \$6.

This supplement to the British Veterinary Codex 1953 brings this excellent publication up-to-date in relation to the B. P. 1958 and B. P. C. 1959. It includes 47 new monographs on drugs, chemicals, and related substances; antisera and vaccines; and 35 additions to the formulary section. A notice on B. V. C. 1953 appeared in THIS JOURNAL, 43, 317(1954).

*Handbuch der Papierchromatographie*. Vol. 1. Grundlagen und Technik. Vol. 2. Bibliographie und Anwendungen. Edited by I. M. HAIS and K. MACEK. Veb Gustav Fischer Verlag, Jena, Germany, 1960. Vol. 1, xxiv + 860 pp. and Vol. 2, xxiv + 728 pp. 16.5 × 24 cm. Price Vol. 1, DM 58.40; Vol. 2, DM 44.

Both volumes are divided into two main sections under General part and Specific applications. Volume 1 has a third section devoted to reagents, materials, and special methods. The books represent a comprehensive treatise on the subject. An English translation would be very useful.

*Biochemical Preparations*. Vol. 7. Edited by HENRY A. LARDY. John Wiley & Sons, Inc., 440 Fourth Ave., New York 16, N. Y., 1960. ix + 102 pp. 15 × 23 cm. Price \$5.25.

Compounds included in Vol. 7 are: N-acetylneuraminic acid, Adenoside di- and triphosphates ( $P^{32}$ ) (chemical and enzyme syntheses),  $\beta$ -Aminoisobutyric acid, Carbamyl phosphate, (—)-Carnitine chloride, Cerebrosides, Coproporphyrin III tetramethyl ester, 6-Deoxy-6-fluoro-D-glucose, dihydroxyacetone phosphate, Flavin adenine dinucleotide (FAD), D-Fructose 1-phosphate (barium salt), 3-Hydroxy-L-kynurenine, Hydroxypyruvic acid phosphate, Methylmalonic semialdehyde, Potassium dihydrogen Ls(+)-isocitrate, S-Palmityl coenzyme A, Stearic acid, methyl stearate, and homologous compounds, Stigmasterol, Tetrahydrofolic acid (5,6,7,8-tetrahydropteroylglutamic acid).

*Chemotherapy in Emotional Disorders*. By FREDERIC F. FLACH and PETER F. REGAN. McGraw-Hill Book Co., Inc., 330 West 42nd St., New York 36, N. Y., 1960. xiv + 314 pp. 15 × 23 cm. Price \$10.

The psychotherapeutic use of somatic treatments is systematically discussed so as to increase the effectiveness with which clinicians in psychiatry and other fields of medicine employ physiological treatments. Particular emphasis is given to the newer psychopharmacologic agents used in the treatment of patients with various emotional disturbances. A glossary of drug names, a bibliography of important books and articles related to the text, and an index are appended.

# AUTHOR INDEX

## A

- Ahdalla, A. M., see Barakat, M. Z., 360  
 Abreu, B. E., see Weaver, L. C., 298  
 Aceto, M. D. G., and Ichimowski, C. T., rat bioassay of combinations of diuretics, 647  
 —see Lynch, V. D., 205  
 Afonso, A., see Kupchan, S. M., 242  
 Allard, K. R., see Dunham, N. W., 218  
 Amer, S. M., see Busch, H., 16  
 Ames, S. R., Swanson, W. J., and Lehman, R. W., estimation of the biological potency of isomerized vitamin A palmitate in aqueous multivitamin dispersions from maleic values, 366  
 —see Lehman, R. W., 363  
 Amundson, M. E., see Lange, W. E., 322  
 Anselmo, C., see Marcus, S., 616  
 Athalye, M. Y., see Guttman, D. E., 687  
 Autian, J., see Goyan, J. E., 627  
 —see Kapadia, A. J., 380  
 —see Kim, H. K., 227  
 —see Udani, J. H., 376

## B

- Bahkian, J. M., see Hamor, G. H., 283  
 Bandelin, F. J., and Tuschhoff, J. V., paper chromatography of some certified dyes, 302  
 Banker, G. S., see Foerzler, E. C., 249  
 —see Peck, G. E., 75  
 Barakat, M. Z., Shehah, S. K., and Abdalla, A. M., antioxidants. The microdetermination of hydroquinone, 360  
 Barnard, R. W., see Pratt, R., 643  
 Barnes, T. C., relationship of chemical structure to central nervous system effects of tranquilizing and anticonvulsant drugs, 415  
 Barr, M., see Stolar, M. E., 144, 148  
 Bartell, P., Pierzchala, W., and Tint, H., adsorption of enteroviruses by activated attapulgite, 1  
 Bauer, C. W., and Lassala, E. F., preparation and the properties of an aspirin homolog  $\beta$  (o acetoxyphenyl) propionic acid, 48  
 Bayeja, S. K., see Gaid, K. N., 659, 663  
 Beal, H. M., see Singiser, R. E., 478, 482  
 Beal, J. L., see Kupchan, S. M., 727  
 Beckman, S. M., preparation and properties of new gastric antacids I. Aluminum hydroxide magnesium carbonate dried gels, 191  
 —and Vogel, C. H., preparation and properties of new gastric antacids II. Aluminum hydroxide protein dried gels, 201  
 Belcastro, P. F., see Fenn, G. D., 105  
 Bell, F. K., digitals X. The infrared absorption spectra of some digitals glycosides and aglycones, 277  
 Bhatia, M. C., see Chopra, C. L., 780  
 Bianculi, J. A., see Bickerton, R. K., 183  
 —see Buckley, J. P., 586  
 —see Farnsworth, N. R., 589  
 Bickerton, R. K., Jacquart, M. L., Kinard, W. J., Jr., Bianculi, J. A., and Buckley, J. P., evaluation of certain hypotensive agents III. Tetrahydroisoquinoline and tetrahydroquinoline derivatives, 183  
 —see Buckley, J. P., 586  
 Bieleke, T., see Vanecek, J., 178  
 Billman, J. H., see Weinberg, E. D., 441  
 Bland, W. H., see Tuhis, M., 422  
 Blake, M. I., quantitative determination of free menthol in peppermint oil, 175  
 —and Rabyohn, G., determination of eucalyptol by residual titration with hydrogen bromide in acetic acid, 650  
 Bolton, S., interaction of citrate with aspirin and benzoic acid, 237  
 Borovansky, A., Sekera, A., and Vrba, C., studies on local anesthetics XXII. The basic aroxy and aralkoxyacetanilides, 57  
 Bose, B. C., Vijayavargiya, R., Saifi, A. Q., and Sharma, S. K., some aspects of chemical and pharmacological studies of *Acorus calamus* Linn., 32  
 Bousquet, W. F., and Christian, J. E.,

- metabolism and central nervous system distribution of  $C^{14}$  carbonyl salicylamide in the rat, 389, note on the synthesis of  $C^{14}$  carboxyl salicylic acid by the halogen-metal interconversion reaction, 406  
 Brady, L. R., and Tyler, V. E., Jr., note on the biosynthesis of clavine alkaloids in *Claviceps purpurea* strain 15B, 332  
 Breckinridge, C. E., Jr., and Christian, J. E., note on the inverse isotope dilution analysis of salicylic acid, 330  
 Brewer, J. H., and Bryant, H. H., toxicity and safety testing of disposable medical and pharmaceutical materials, 652  
 Brown, B. T., and Wright, S. E., absorption spectra of cardiac glycosides and aglycones in sulfuric acid, 777  
 Brummett, R. E., and Scuchetti, L. A., changes induced by gibberellic acid on growth and alkaloid patterns in *Datura stramonium* L. and in *Atropa belladonna* L., 274  
 Bruno, G. A., and Christian, J. E., note on suitable solvent systems usable in the liquid scintillation counting of animal tissues, 560  
 Bryant, H. H., see Brewer, J. H., 652  
 Buckley, J. P., Jacquart, M. L., Bickerton, R. K., Hudak, W. J., Schalit, F. M., DeFeo, J. J., and Bianculi, J. A., evaluation of certain hypotensive agents IV. Diquaternized piperidine derivatives, 586  
 —see Bickerton, R. K., 183  
 Burkman, A. M., note on the characteristics of an apomorphine response in pigeons, 558  
 Busch, H., Amer, S. M., and Davis, J. R., antitumor activity of sodium dichloropyruvate, 16  
 Busse, L. W., see Finger, K. F., 569  
 —see Strickland, W. A., Jr., 35  
 Butler, C. L., see Youngken, H. W., Sr., 271

## C

- Caldwell, H. C., communication color selection for uncoated tablets, 624  
 Camp, B. J., and Moore, J. A., quantitative method for the alkaloid of *Acacia berlandieri*, 158  
 Campbell, J. A., see Morrison, A. B., 473  
 Carkhuff, E. D., see Tuerck, P. A., 344, 347  
 Chaykin, L., see Forlano, A. J., 67  
 Cheng, S.-S., and Jonsson, S., synthesis of  $\beta$  aminoethyl ketones as potential antagonists of  $\beta$  alanine, 611  
 Chermun, R., see Weinberg, E. D., 441  
 Chertkoff, M. J., and Martin, A. N., solubility of benzoic acid in mixed solvents, 444  
 Chong, C. W., Eriksen, S. P., and Swintsky, J. V., antithyrotropic behavior of magnesium magma, 547  
 —see Reese, D. R., 85  
 Chopra, C. L., Bhatia, M. C., and Chopra, I. C., *in vitro* anthrathic activity of oils from Indian medicinal plants I, 780  
 Chopra, I. C., see Chopra, C. L., 780  
 Christian, J. E., see Bousquet, W. F., 389, 406  
 —see Breckinridge, C. E., Jr., 330  
 —see Bruno, G. A., 560  
 —see O'Malley, W. J., 398, 402  
 —see Redman, G. D., 98  
 —see Schiffman, R. F., 59  
 Chulski, T., see Hamlin, W. E., 253  
 Ciaccio, L. L., see Missan, S. R., 7  
 Cohen, E. M., see Lord, N. G., 371  
 Cohen, S., see Young, J. G., 72  
 Coker, S. T., see Joseph, (Sister Daniel), 101  
 Compeau, G. M., adsorption of dodecyl benzenesulfonate and hexachlorophene on the skin, 574  
 Cooper, J., see Lachman, L., 165, 213  
 Counsell, R. E., and Some, T. O., esters of bicyclic aminoalcohols II. The synthesis of the hydroxyquinoxalidines

- and some of their esters as potential therapeutic agents, 289  
 Counter, F. T., Jr., Duval, R. N., Foye, W. O., and Vanderwyk, R. W., preparation and antibacterial action of metal chelates of some antitubercular agents, amino acids, and peptides, 140  
 Crane, F. A., see Schermeister, L. G., 694, 698

## D

- Davis, J. R., see Busch, H., 16  
 DeFeo, J. J., see Buckley, J. P., 586  
 Deitz, W. H., see McChesney, E. W., 762  
 DeKay, H. G., see Peck, G. E., 75  
 Delahunt, C. S., see Finkelstein, M., 18  
 DeLuca, P. P., and Kostachauder, H. B., interaction of preservatives with macromolecules IV. Binding of quaternary ammonium compounds by nonionic agents, 130  
 —see Souder, J. C., 255  
 Demetrius, J. C., Jr., and Sinsheimer, J. E., application of  $\Delta\epsilon$  analysis to pharmaceuticals: the determination of eugenol, 523  
 Dempski, R. E., see Wurster, D. E., 305  
 de Ropp, R. S., chromatographic separation of the phenolic compounds of *Cannabis sativa*, 756  
 Dieterle, J. M., see Lehman, R. W., 363  
 Domer, F. R., and Schueler, F. W., synthesis and metabolic studies of  $C^{14}$ -labeled hemicholinum No. 3, 553  
 Doorenhos, N. J., see Havranek, R. E., 328  
 Draus, F. J., see Farnsworth, N. R., 589  
 Duerr, J. D., and Pappas, B. A., determination of prednisolone in the presence of hydroxyzine and in formulations containing analgesics, 759  
 Dunham, N. W., and Allard, K. R., preliminary pharmacologic investigation of the roots of *Bixa orellana*, 218  
 Duval, R. N., see Counter, F. T., Jr., 140

## E

- Eckert, H. W., see McChesney, E. W., 28  
 Eiler, J. J., see Singer, W., 90, 669  
 Eisenhardt, L. L., see Joseph, (Sister Daniel), 101  
 Elliott, D., see Sciarra, J. J., 115  
 El-Marrah, A., see Osman, H. G., 231  
 Epstein, E., and Malatestic, N., synthesis and pharmacology of N-(substituted aminoacyl)-chlorotoluidines, 11, 80  
 Eriksen, S. P., Irwin, G. M., and Swintsky, J. V., heating and cooling rate coefficients and related factors affecting procedures for tablet shelf life prediction, 632  
 —see Chong, C. W., 547  
 Esch, B., and Schaeffer, H. J., note on the synthesis of dihydroresorcinol, 786  
 Evanson, R. V., see Smith, H. A., 94

## F

- Fand, T. I., see Vidal, F., 535  
 Farnsworth, N. R., Draus, F. J., Sager, R. W., and Bianculi, J. A., studies on *Vinca major* L. (*Apocynaceae*) I. Isolation of perivincine, 589  
 Fenn, G. D., and Belcastro, P. F., investigation of the effect of ultrasonic waves on the rates of hydrolysis of procaine and butethamine hydrochlorides, 105  
 Finger, K. F., Lemberger, A. P., Higuchi, T., Busse, L. W., and Wurster, D. E., investigation and development of protective ointments IV. The influence of active fillers on the permeability of semisols, 569  
 —Lemberger, A. P., Wurster, D. E., and Higuchi, T., investigation and development of protective ointments III. Adsorption characteristics of saria from solutions, 565  
 Fink, G. B., and Swinyard, E. A., effects of psychopharmacologic agents on

- experimentally-induced seizures in mice, 510
- Finkelstein, M., Kromer, C. M., Sweeney, S. A., and Delahunt, C. S., some aspects of the pharmacology of clemizole hydrochloride, 18
- Fischer, L., see Pettinato, F. A., 45
- Fisher, W. T., see Lehman, R. W., 363
- Foernzler, E. C., Martin, A. N., and Banker, G. S., effect of thixotropy on suspension stability, 249
- Forlano, A. J., and Chavkin, L., effect of granule size upon disintegration time and capping in compressed tablets 67
- and Harris, L. E., effects of fatty acids on vitamin A esters in isopropanol solutions 451, preparation and stability of some esters of vitamin A 458
- Foye, W. O., and Kay, D. H., preparation of 1-alkyl-2,5 piperazinediones 705
- see Counter, F. T. Jr., 140
- see Pecci, J., 411
- Freedman, L., see Shapiro, S. L., 737

## G

- Gaind, K. N., and Baveja, S. K., investigation of *Machilus macrantha* Nees I Pharmacognostical and phytochemical study of root 659, investigation of *Machilus macrantha* Nees II Pharmacological action and chemical constitution of machiline 663
- Gainor, C., see Simmons, R. J., 63
- Garrett, E. R., prediction of stability in pharmaceutical preparations VII The solution degradation of the antibiotic streptomycin, 767
- and Hanka, L. J., psicofuramine correlation of assay methods in acid degradation studies 526
- see Tingstad, J. E., 352
- Gass, L. D., and Martin, J. W. Jr., note on esters of  $\beta$ -phenyl  $\alpha$ -benzyllox iminopropionic acid, 405
- Gatewood, L. J., and Graham, H. D., adaptation of the chromotropic acid method to the assay of Spans 678
- Gerraughy, R. J., and Jannke, P. J., esterification of two sterically hindered acids using ultrasound waves 350
- Getzkin, A. J., and Lauter, W. M., synthesis of some symmetrical aldehyde glycol monoether acetals 746
- Gibson, R. D., see Malone, M. H., 529
- Givold, O., see Ormrod, G., 153
- Gjerstad, G., see Gonzalez, E. E., 782
- Goldberg, M. E., and Rossi, G. V., effect of anticholinergic compounds on several components of gastric secretion in pylorus ligated rats 543
- Goldberg, M. M., and Mann, D. E. Jr., antidotal effectiveness of sodium cobaltinitrite in antagonizing cyanide poisoning in albino mice 210
- Gonzalez, E. E., and Gjerstad, G., metabolic and morphological changes induced by gibberellic acid on spearmint 782
- Goudie, A. J., see Scott, M. W., 467
- Goyan, J. E., note on improvement of separations in paper partition chromatography, 408
- Shaikh, Z. I., and Autian, J., kinetic study of barbitol degradation in an ammonia buffer system 627
- Graham, H. D., see Gatewood, L. J., 678
- Greenberg, L., and Ingalls, J. W., effects of tranquilizers on bacterial toxemias II Meprobamate, 637
- Ingalls, J. W., and Zupko, A. G., effects of tranquilizers on bacterial toxemias I Reserpine, 225
- and Tirpak, J., note on the effect of gibberellic acid on *Azotobacter indicus*, 333
- Grenfell, T. C., see Missan, S. R., 7
- Guess, W. L., note on the hydrophile lipophile balance of tragacanth, 736
- Hall, N. A., and Rusing, L. W., gum from rain lily, *Cooperia pedunculata*, 102
- Guttman, D. E., and Athalye, M. Y., solubilization of riboflavin by complex formation with caffeine, theophylline, and dimethyluracil, 687

## H

- Haddad-Louis, W. A., and Lemberger, A. P., investigation of particle-medium interactions in suspensions, 463
- Hall, N. A., see Guess, W. L., 102
- see Pettinato, F. A., 45
- see Roscoe, C. W., 108
- see Wood, J. A., 180
- Hamlin, W. E., Chulski, T., Johnson, R. H., and Wagner, J. G., note on the photolytic degradation of anti-inflammatory steroids, 253
- Hamor, G. H., saccharin derivatives II Synthesis of 4-nitrosaccharin and related compounds, 280
- and Bahkian, J. M., saccharin derivatives III A note on the synthesis of hisaccharins, 283
- Hanka, L. J., see Garrett, E. R., 526
- Hanna, C., preparation of tritium-labeled haloethane (2-bromo-2-chloro-1,1,1-trifluoroethane) 502
- Harris, L. E., see Forlano, A. J., 451, 458
- see Lamb, D. J., 583
- see Leyda, J. P., 581
- Hartman, C. W., see Rhynce, J. W., 234
- see Spittle, R. Y., 325
- Havemeyer, R. N., and Higuchi, T., complexing tendencies of cyanocobalamin with inorganic compounds Heteromolybdates and heavy metal chlorides, 356
- Havranek, R. E., and Doorenhos, N. J., steroids I Synthesis of steroid nitrogen mustards 328
- Haycock, R. P., Sheth, P. B., and Mader, W. J., quantitative fluorometric reaction for glutethimide, 673
- Hayden, A. L., and Sammul, O. R., infrared analysis of pharmaceuticals I Application of the potassium bromide disk technique to some steroids alkaloids, barbiturates, and other drugs, 489, infrared analysis of pharmaceuticals II A study of the cinchona alkaloids in potassium bromide disks, 497
- Heimlich, K. R., and Martin, A. N., kinetic study of glucose degradation in acid solution, 592
- Higuchi, T., see Finger, K. F., 565, 569
- see Havemeyer, R. N., 356
- see Higuchi, W. I., 598
- see Salishury, R., 284
- see Schroeter, L. C., 331
- see Strickland, W. A., Jr., 35
- Higuchi, W. I., and Higuchi, T., theoretical analysis of diffusional movement through heterogeneous barriers 598
- Hiner, L. D., see Smith, D. L., 538
- see Workman, R. L., Jr., 118
- Hopkins, H., and Small, L. D., investigation of some pharmaceutical applications of certain fatty acid esters of sucrose, 220
- Hopkinson, A. F., see Pachter, I. J., 621
- Hubscher, M. H., yellow phenolphthalein, 308
- Hudak, W. J., see Buckley, J. P., 586
- Huetteman, A. J., see Scott, M. W., 467
- Husa, W. J., see Winters, E. P., 709

## I

- Iehnowski, C. T., see Aceto, M. D. G., 617
- Ingalls, J. W., see Greenberg, L., 225, 637
- Irwin, G. M., see Eriksen, S. P., 632

## J

- Jacquert, M. L., see Bickerton, R. K., 183
- see Buckley, J. P., 586
- James, A. E., see Klein, S., 311
- Jannke, P. J., see Gerraughy, R. J., 350
- Jenkins, H. J., see Youngken, H. W., 271
- Johnson, R. H., see Hamlin, W. E., 253
- Jonsson, S., see Cheng, S. S., 611
- Joseph, (Sister Daniel), Coker, S. T., and Eisenbrandt, L. L., effect of hydration on hydrocholeresis in rats, 101
- Judis, J., photo oxidation of dihydroxyphenylalanine in the presence of 8-methoxypsoralen, 117

## K

- Kapadia, A. J., and Autian, J., study of the stability of secobarbital sodium solutions II Separation and identification of degradation products of secobarbital sodium, 380
- Kay, D. H., see Foye, W. O., 705
- Khastigir, H. N., Sengupta, S. K., and Sengupta, P., note on the constituents of the Indian medicinal plant *Oldenlandia corymbosa* Linn., 562
- Kier, L. B., and Some, T. O., alkaloids of *Argemone munita* subsp. *rotundata*, 187
- Kim, H. K., and Autian, J., binding of drugs by plastics II Interaction of weak organic acids with plastic syringes 227
- Kinard, W. J., Jr., see Bickerton, R. K., 183
- Klein, S., James, A. E., and Tuckerman, M. M., spectrophotometric assay for combinations of ethinyl estradiol and methyltestosterone, 314
- Koss, R. F., see McChesney, E. W., 28, 762
- Kostenhauder, H. B., see DeLuca, P. P., 430
- Krebs, V., see Vanceek, J., 178
- Kroeger, D. C., and Lucco, L. J., scrotoin induced apnea, 170
- Kromer, C. M., see Finkelstein, M., 18
- Kruger-Thiemer, E., dosage schedule and pharmacokinetics in chemotherapy, 311
- Kuhlik, E., see Wagner, J. G., 133
- Kupehan, S. M., and Afonso, A., veratrum alkaloids XLIII The structure of cevadine, 242
- and Ohasi, M. E., note on the occurrence of 2,6-dimethoxybenzoquinone in *Rauwolfia tomentosa*, 257
- Yokoyama, N., and Beal, J. L., menispermaceae alkaloids I The alkaloids of *Cissampelos pareira* Linn and the origin of *radix paveriae bravae*, 727

## L

- Lachman, L., Swartz, C. J., and Cooper, J., comprehensive pharmaceutical stability testing laboratory III A light stability tunnel for evaluating the photosensitivity of pharmaceuticals, 213
- Swartz, C. J., Urbanyi, T., and Cooper, J., color stability of tablet formulations II Influence of light intensity on the fading of several water-soluble dyes, 165
- see Urbanyi, T., 163
- Lamb, D. J., and Harris, L. E., correlation of the distribution coefficients of various barbituric acids, 583
- see Leyda, J. P., 581
- Lange, W. E., McMurtry, J., and Amundson, M. E., metal chelates and diabetogenic activity II Isoalloxazines, 322
- Lasala, E. F., see Bauer, C. W., 18
- Lauter, W. M., see Getzkin, A. J., 716
- Lawrence, C. A., antimicrobial activity, *in vitro*, of chlorhexidine, 731
- Leary, J. B., determination of basic epoxides, 606
- Lee, K.-H., action of ethyl carbamate on oxidative phosphorylation, 609
- see Singer, W., 90
- Lehman, R. W., Dieterle, J. M., Fisher, W. T., and Ames, S. R., isomerization of vitamin A in aqueous multivitamin drop preparations, 363
- see Ames, S. R., 366
- Lemberger, A. P., see Finger, K. I., 565, 569
- see Haddad-Louis, W. A., 163
- see Marvel, J. R., 117, 120
- Levy, G., and Schwarz, T. W., study of aqueous medicinal lubricants, 504
- Leyda, J. P., Lamb, D. J., and Harris, L. E., distribution coefficients and dissociation constants of a series of barbituric acid derivatives 581
- Long, S., see Wagner, J. G., 121, 128, 133
- Lord, N. G., Cohen, I. M., and Taylor, B. L., application of alternating current polarography in the determina-

